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The role of telomeres and chromosome linearity in the survival of DNA damage

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I, Anna Katherine Hebden, confirm that the work presented in this thesis is my own. Where information has beem derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The DNA at the ends of most eukaryotic chromosomes is comprised of repetitive sequences packaged with proteins into structures called telomeres. These complexes differentiate natural chromosome ends from those of DNA breaks and therefore play an important role in maintaining genome integrity. Telomerase maintains telomeres by the addition of DNA repeats to chromosome ends. Disruption of this enzyme leads to loss of telomeric DNA with successive rounds of DNA replication, and in most cases, senescence. Following loss of Trt1, the catalytic subunit of telomerase in fission yeast, a population of cells survive having lost all telomeric DNA. These cells survive by maintaining each of the three chromosomes as individual circles.

Reminiscent of strains lacking the telomere binding protein Taz1, circular strains are hypersensitive to agents that induce DNA double strand breaks. Here we present our data into the further understanding of the role telomeres play in survival following damage.

A partial suppression of sensitivity is observed upon linearisation of a single chromosome. To further distinguish between the topological issue of chromosome circularity and the presence or absence of telomere sequence, we created strains containing telomere repeats lacking ends, either plasmid based (to allow high copy number) or integrated within the genome. In most cases the presence of telomere repeats did not affect the drug sensitivity. Intriguingly, however, we observed rare survivors with greatly suppressed drug sensitivity upon disruption of telomerase. Analysis shows these strains have survived by a novel mechanism. While they appear to lack the majority of telomeric DNA, they show behaviours distinct from those of typical circular chromosome-containing survivors. Our data suggest that one of these strains survives by amplifying subtelomeric repeat sequences, and the other by amplifying rDNA sequences. These strains have aided us in our understanding of the role telomeres play in survival following genotoxic insult.

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Thank you!

Contents

List of tables and figures

Abbreviations

5'-FOA	5'-fluorootic acid
°C	degrees Celsius
γ-irradiation	gamma irradiation
μl	microliter
μM	micromolar
μJ	microjoules
∞	forever
A	Adenine
ALT	alternative lengthening of telomeres
APB	ALT-associated premyelocytic leukaemia body
BIR	break induced replication
bp	base pairs
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
ch	chromosome
CHEF	contour-clamped homogeneous electric field
CPT	camptothecin
C-terminal	carboxyl terminal
DAPI	4', 6-diamidino-2-phenylindole
dATP	deoxyadeenosine 5'-triphosphate
dCTP	deoxycytosine 5'-triphosphate
ddH_2O	double distilled water
DIC	differential interference contrast
D-loop	displacement loop
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide 5'-triphosphate
DSBs	double strand breaks
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid

EMM	Edinburgh minimal medial
EtOH	ethanol
FACS	fluorescence activated cell sorter
g	grams
G	guanine
G1	gap phase 1
G2	gap phase 2
GFP	green fluorescent protein
Gy	grays
HCl	hydrochloric acid
HR	homologous recombination
hrs	hours
HU	hydroxyurea
J	joules
Kan	kanamycin
Kb	kilobases
LiOAc	lithium acetate
LMP	low melting point
LTR	long terminal repeat
m	meter
M	molar
Mb	megabase
mg	milligram
min	minute
ml	mililiter
MMS	methylmethane sulfonate
MOPS	3-(N-morpholino)propanesulfonic acid
M-phase	mitosis
MRN	Mre11/Rad50/Nbs1
MRX	Mre11/Rad50/Xrs2
N	nitrogen
ng	nanogram
NHEJ	non-homologous end joining
N-terminal	amino terminal
nt	nucleotide

OB	oligonucleotide/oligosaccharide binding
Oligo	oligonucleotide
PCR	polymerase chain reaction
PEG4000	polyethyleneglycol 4000
PFG	pulsed field gel
PFGE	pulsed field gel electrophoresis
PML	premyelocytic leukaemia body
PMSF	phenylmethylsulphonyl fluoride
^{r}	resistance
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
RNase	ribonuclease
SAC	spindle assembly checkpoint
SDS	sodium dodecyl sulfate
SIN	septation initiation network
SP1	spheroplast buffer
SPB	spindle pole body
S-phase	synthesis phase
SSC	saline sodium citrate buffer
STE	subtelomeric element
T	thiamine
TAE	tris-acetate + EDTA
Taq	*Thermus aquaticus*
TBE	tris-borate + EDTA
TBZ	thiabendazole
TE	tris + EDTA
T-loop	telomeric loop
Tm	melting temperature
TPE	telomere position effect
TRD	telomere rapid deletion
ts	temperature sensitive
u	units
UV	ultraviolet
X	times
YE4S	yeast extract media + supplements

1 Introduction

1.1 Telomeres

Most eukaryotic chromosomes are organised into linear structures and therefore have physical ends. The term telomere, from the Greek *telos* meaning "end" and *meros* meaning "part", describes the terminal structure of chromosomes. In the late 1930's, while studying X-ray induced breaks in chromosomes, Hermann Muller noted that, while broken chromosome ends rejoin with other breaks, this never involved the natural ends of chromosomes. Terminal inversions or deletions were never recovered. He proposed that essential structures, or 'terminal genes' were present, capping natural chromosome ends and excluding them from these rejoining reactions (Muller, 1938). Around the same time, Barbara McClintock made a similar observation while studying meiotic chromosomes in maize. Chromosomes broken during meiosis were involved in cycles of fusion with other broken chromosomes forming dicentrics, followed by asymmetric breakage, leading to the loss of some genetic material in some cells and duplication in others. Intact chromosomes, however, were somehow protected from the so-called 'breakage-fusion-bridge' cycles by the presence of terminal protective structures (McClintock, 1938). The telomere was discovered.

It is becoming ever more apparent that the structure and function of telomeres and proteins involved in their regulation is very complex and dynamic. Protein associations with telomeres and telomere structures may vary with the cell cycle or under different conditions. The telomere complex plays a diverse role in the cell, ranging from allowing replication of linear DNA molecules to evasion of activation of a DNA damage response by the natural chromosome ends to roles in meiosis. Telomeres also may play a role in the survival of cells following DNA damage. The complexity of telomere dynamics was recently demonstrated through a screen carried out in budding yeast, looking for deletion strains that affect telomere length. Over 150 viable deletions with previously unrecorded telomere length alterations were uncovered (Askree et al., 2004). Clearly a complete documentation of telomere biology would be unattainable and unnecessary for this work, so in this chapter I shall give an

overview of telomere biology, comparing fission yeast, budding yeast and mammals, referring to other examples where appropriate, and focusing on the areas that are most relevant to my work.

1.2 Telomere structure

In this section, I will discuss the structure of the telomeric DNA and its basic associations with some of the main proteins that form the complex. Telomeres of mammals and yeast are first discussed as an overview of the common telomere complex in eukaryotic cells. *Drosophila* telomeres are then discussed as an exception to the general rules of telomere structure. Figure 1.1 shows a schematic representation of the telomeres in humans, budding and fission yeasts and *Drosophila* and can be used as a reference.

1.2.1 Mammalian and yeast telomeres

In most species, the DNA at telomeres consists of short, guanine rich sequences arranged into tandem repeats. In vertebrates the repeat sequence is 5' TTAGGG 3' (Moyzis et al., 1988) and in yeast a related, but degenerative sequence is used (5' $TG_{1\text{-}3}$ 3' in budding yeast (Shampay et al., 1984) and '5 $TTACAG_{1\text{-}8}$ 3' in fission yeast (Hiraoka et al., 1998)). Telomere DNA varies in length between organisms from about 300bp in yeast (Shampay et al., 1984; Sugawara, 1989) to 5-15kb in human cells (Allshire et al., 1989; de Lange et al., 1990) and 60kb in laboratory mice (Kipling and Cooke, 1990; Starling et al., 1990; Zijlmans et al., 1997). Running 5' to 3' towards the terminus, the G-rich strand overhangs its complementary strand (Henderson and Blackburn, 1989). The overhang also varies in length between organisms from around 150 nucleotides in humans (Huffman et al., 2000; Wright et al., 1997) to 12 nucleotides in budding yeast (Shampay et al., 1984). In yeast and some other organisms telomeres are usually found in clusters at the nuclear periphery (Funabiki et al., 1993; Gotta et al., 1996). However, in mammalian cells this is not observed (Luderus et al., 1996; Vourc'h et al., 1993). Proteins may bind either the single or double strand regions of DNA and may also recruit other proteins to the complex. The proteins play an important role in telomere regulation, both in the length homeostasis, structure and function of the telomere. Some organisms show exceptions to the usual telomere structure; chromosome ends may be

comprised of different sequences and maintained by different mechanisms, but their basic functions remain conserved. These exceptions will be discussed later in the chapter.

As will be discussed in greater detail in Chapter 1.3.3, one role of telomeres is to allow complete replication of the chromosome ends, thereby overcoming the end replication problem. Telomere DNA is added onto chromosome ends by the action of a ribonucleoprotein complex, telomerase. The telomerase holoenzyme consists of a reverse transcriptase catalytic subunit, an RNA moiety used as a template to copy DNA to the chromosome ends, and accessory proteins that regulate the action of the enzyme. In fission yeast, the catalytic subunit, Trt1, which contains the reverse transcriptase activity of the enzyme, shows 30% homology to hTERT, the human counterpart (Nakamura et al., 1997). Disruption of *trt1*$^{+}$ leads to telomere shortening with successive rounds of cell division and eventual cell cycle arrest (Nakamura et al., 1998). However, as discussed later in this chapter, survivor populations may emerge (Nakamura et al., 1998). Despite the activity of telomerase being RNase sensitive, the RNA subunit has yet to be found in fission yeast. A telomerase accessory factor, Est1, has also been identified in fission yeast. As with budding yeast, Est1 is required for telomerase processivity *in vivo* but not *in vitro*. Disruption leads to a phenotype reminiscent of a *trt1*$^{+}$ deletion (Beernink et al., 2003). Budding yeast telomerase is comprised of the catalytic subunit, Est2, the RNA template, TLC1 and accessory factors EST1 and EST3 (Lendvay et al., 1996; Lundblad and Szostak, 1989; Singer and Gottschling, 1994). In humans, the catalytic subunit is encoded by *hTERT* and the RNA moiety by *TR*. In most somatic cells, telomerase is not active due to lack of the catalytic subunit (Bodnar et al., 1998).

The mammalian telomere recruits two main double stranded DNA binding proteins, TRF1 and TRF2 (Bilaud et al., 1997; Broccoli et al., 1997; Zhong et al., 1992). TRF1 is involved in telomere length regulation, and TRF2 in end capping. Fission yeast has a single homologue of the two mammalian proteins, Taz1 (Cooper et al., 1997). Both Taz1 and the TRF proteins contain a C-terminal domain that is related to the DNA binding domain of Myb transcriptional activators (Broccoli et al., 1997; Cooper et al., 1997). At the N-

terminal region, the proteins share a 'TRF homology' domain, thought to be involved in protein-protein interactions, both as homodimers and heterodimers (Li et al., 2000). Taz1 is involved in regulating telomere length and formation of the heterochromatin domain. Disruption leads to a 10-fold increase in telomere length and loss of the telomere position effect (Cooper et al., 1997). Despite the obvious deregulation of telomere structure in *taz1*Δ mutants, growth under optimal vegetative conditions is normal. However, when growth conditions are altered, the important roles of telomeres and the role of Taz1 in telomere function become more apparent (Cooper et al., 1998; Ferreira and Cooper, 2001; Miller and Cooper, 2003). These situations will be discussed in greater detail later in the chapter (Chapter 1.3.2.2).

Unlike in fission yeast and mammals, budding yeast does not have a TRF like telomere binding protein. The major telomere protein is the Repressor-activator protein, RAP1 which, unlike fission yeast Rap1, binds directly to telomere repeats (Konig et al., 1996; Konig and Rhodes, 1997). Budding yeast RAP1 also functions as a transcription factor at other areas of the genome complicating analysis of RAP1 function at telomeres difficult. It is involved in telomere length regulation at telomeres; over-expression of RAP1 leads to an increase in telomere length, and temperature sensitive mutants show telomere shortening when grown at a semi-permissive temperature (Conrad et al., 1990). Similarly, a C-terminal truncation mutant shows a deregulation of telomere length (Kyrion et al., 1992). This has been attributed to the C-terminus of RAP1 being involved in a 'counting mechanism', with the length of the telomere being proportional to the number of RAP1 molecules bound (Marcand et al., 1997). RAP1 also recruits other telomere-associated proteins to the complex, such as RIF1 and RIF2, also involved in telomere length regulation (Wotton and Shore, 1997). SIR proteins also interact with RAP1, nucleating a heterochromatin domain and causing the repression of genes placed adjacent to telomeres (Moretti et al., 1994).

Both mammals and fission yeast do have Rap1 homologues, however these proteins do not bind directly to telomere DNA. Instead, they are recruited to the telomeres via the TRF proteins or Taz1 respectively (Kanoh and Ishikawa, 2001; Li et al., 2000). Taz1 also acts to recruit Rif1 to the telomere complex

(Kanoh and Ishikawa, 2001). Recruitment of these proteins to the telomeres is required for telomere function, suggesting Taz1 acts, at least in part, through its recruitment of other proteins to the telomere complex (Kanoh and Ishikawa, 2001; Miller et al., 2005). Human Rif1 does not associate with telomeres and its deletion does not confer a telomere phenotype (Silverman et al., 2004). The human TRF proteins also recruit TIN2 to the telomere complex (Houghtaling et al., 2004; Kim et al., 1999; Li et al., 2000), which interacts with Pot1 via an interaction with TPP1 (Ye et al., 2004b).

While telomerase action contributes to generation of the 3' single stranded overhang (see Chapter 1.3.3), an important structure of the telomere, is not the only important step. Incomplete synthesis of the 5' strand by lagging strand replication with or without telomerase-mediated synthesis of the 3' strand, coupled with removal of the RNA primer of the terminal Okasaki fragment required for lagging strand replication would both lead to a 3' overhang. However, there is strong evidence to suggest much of the overhang length is produced by endonuclease degradation of the 5' strand (Huffman et al., 2000; Larrivee et al., 2004; Makarov et al., 1997). In budding yeast, generation of the overhang is dependent on the activity of the MRX complex (Larrivee et al., 2004). Little is known about the overhang structure in fission yeast, however *taz1*Δ mutants have an elongated 3' overhang, the generation of which is dependent on both telomerase activity and DNA processing (Tomita et al., 2003). The presence of a 3' overhang structure has been implicated in creating a specialised structure at chromosome ends. The so-called T-loop (telomeric loop) structure would form through protein mediated strand invasion of the single strand overhang into the double strand region. Displacement of a region of the duplex DNA (the D-loop) allows pairing of the single strand overhang with the internal region of telomere DNA. Indeed, electron microscopy on human and mouse telomeres has demonstrated the existence of such looped structures *in vivo* (Griffith et al., 1999). *In vitro* studies of fission yeast telomeres suggests Taz1 is able to promote a T-loop structure (Tomaska et al., 2004). Whether a similar structure can be generated at budding yeast telomeres has yet to be determined. T-loops may act as a means to hide the chromosome ends from inappropriate digestion by nucleases and evasion of the DNA damage

response. Such structures may also be involved in regulation of telomerase activity by limiting the access of telomerase to the chromosome end. Indeed, one might imagine that as telomeres get shorter, the ability to form a loop may be diminished, thereby releasing the end and exposing it for elongation by telomerase.

The single strand overhang is bound by the oligonucleotide/oligosaccharide binding (OB)-fold protein, Pot1 in mammals and fission yeast (Baumann and Cech, 2001) and Cdc13 in budding yeast (Garvik et al., 1995; Lin and Zakian, 1996; Weinert and Hartwell, 1993). Binding of these proteins play important roles in regulation of telomerase action and capping of the chromosome ends. Mammalian Pot1 interacts with another telomere associated protein, TPP1 (also previously known as PTOP, PIP1 and TINT1). TPP1 interacts with the TRF proteins via a bridging protein, TIN2, potentially providing a mechanism for the creation of the T-loop structure (Liu et al., 2004; Ye et al., 2004a; Ye et al., 2004b). TPP1 is important for the recruitment of POT1 to telomeres (Liu et al., 2004; Ye et al., 2004b).

Other proteins that bind or associate with telomeres include a variety of DNA damage and checkpoint proteins, too many to discuss each in detail here (d'Adda di Fagagna et al., 2004; Dahlen et al., 1998; Nakamura et al., 2002; Nugent et al., 1998). Paradoxically, these proteins play an active role in telomere maintenance. These proteins include the checkpoint kinases ATM and ATR, the Ku heterodimer and the MRN complex.

The DNA adjacent to the telomeres is also comprised of specialised sequences know as telomere associated sequences, or subtelomeric elements (STEs), as they will be referred to for the rest of the thesis. In fission yeast, STE are present just centromere-proximal to the telomeres of chromosomes I and II. These STEs comprise about 19 kb of degenerative repeats, split into three regions, STE1-3 (Sugawara, 1989). Chromosome III contains the rDNA repeats at the subtelomeric region of each arm. There also may be STEs between the telomere and rDNA, but this is likely to be strain specific (Sugawara, 1989). The subtelomeric region of budding yeast is

also comprised of repetitive sequences. Two major classes of subtelomeric sequence exist in budding yeast, the X elements and the Y' elements (Chan and Tye, 1983). Human subtelomeric regions are not as well characterised as in yeast. They also have a highly repetitive nature and are thought to stretch from 1kb to more than 200kb in size (Riethman et al., 2004). The best described human STE is D4Z4, due to its implications in the disease, facioacapulohumeral muscular dystrophy (FSHD) (van Deutekom et al., 1993; Wijmenga et al., 1993). STEs may provide some level of end protection in the absence of telomere repeats. Fission yeast surviving in the absence of telomere DNA do so through chromosome circularisation (see Chapter 1.3.4). Interestingly, intramolecular fusion occurs following not only loss of telomere DNA, but also 5-7 kb of STE suggesting there may be some residual protection in the subtelomere region (Nakamura et al., 1998). In budding yeast, survival in the absence of telomerase leads to survival through recombination based mechanisms (see Chapter 1.3.4). One of the survival mechanisms employed involves amplification of STE, maintaining short telomere sequences.

1.2.2 *Drosophila* telomeres: an exception to the rule

The concept of telomeres was first born through the study of the fruit fly, *Drosophila*. However, it has become apparent that *Drosophila* telomeres are an exception to the rule of most other eukaryotes. They maintain chromosome ends in a telomerase independent fashion. Rather than being replicated by telomerase, non-long terminal repeat (LTR) transposable elements act to prevent degradation of chromosome termini (Biessmann et al., 1992; Levis et al., 1993). Two classes of retrotransposible elements have been characterised at *Drosophila* telomeres; *HeT-A* and *TART* (Levis et al., 1993; Rubin, 1978; Young et al., 1983) and a third more recently described, *TAHRE* (Abad et al., 2004). Despite the obvious difference in the mode of telomere maintenance in *Drosophila* compared with the more widely recognised mode in other eukaryotes, there are basic similarities. Both telomerase-derived telomere repeats and those seen in *Drosophila* are added by reverse transcription of an RNA template. This is either the RNA subunit of telomerase or the RNA intermediate of the transposon. The retrotransposons at *Drosophila* telomeres are arranged in tandem repeats and may be

truncated at the 5' end. Interestingly, as with conventional telomere repeats, the DNA running 5' to 3' towards the terminus is also GT rich.

The telomeric transposable elements of *Drosophila* are the only known transposons to have a vital cellular function. They transpose only to chromosome ends, although fragments can be found in the centromeric heterochromatin (Agudo et al., 1999; Biessmann et al., 1992; Levis et al., 1993; Losada et al., 1999; Traverse and Pardue, 1989). The model for transposition to chromosome ends is thus: elements present at the telomere are transcribed and the RNA transported to the cytoplasm where it is translated. Here the RNA is incorporated with the proteins into a ribonuclear protein particle and transported back to the nucleus where it locates to chromosome ends and acts as a template for reverse transcription. The resulting DNA is added to the chromosome ends, lengthening the telomere sequence (Pardue and DeBaryshe, 2003). Transcription of *TART* occurs in both the sense and antisense directions, with the antisense transcripts in 10-fold excess over the sense copies (Danilevskaya et al., 1999). The two transcripts of *TART* could be involved in an RNAi mediated regulation of either the *HeT-A* or both transposable elements' expression. *HeT-A* lacks the reverse transcriptase domain for its own transposition, so this activity must be produced from an alternative, as of yet undefined source (Biessmann et al., 1994). A possibility is that the reverse transcriptase activity of *TART,* or indeed *TAHRE,* also acts on the RNA transcript of *HeT-A.* It is also interesting to observe that *TART* localisation to telomeres is dependent on *HeT-A*, suggesting a possible co-dependence of the two elements (Rashkova et al., 2003). Recombination and gene conversion also seem to play a role in maintaining telomere length in *Drosophila* (Kahn et al., 2000; Mikhailovsky et al., 1999).

The heterochromatin associated protein, HP1, is conserved among many organisms (James and Elgin, 1986; Singh et al., 1991). In *Drosophila* it also localises to telomeres and provides the capping feature of *Drosophila* telomeres. Disruption of the protein causes chromosome end fusions, leading to anaphase bridges and chromosome breakage (Fanti et al., 1998). HP1 not only serves a function in capping *Drosophila* telomeres, but also in regulating

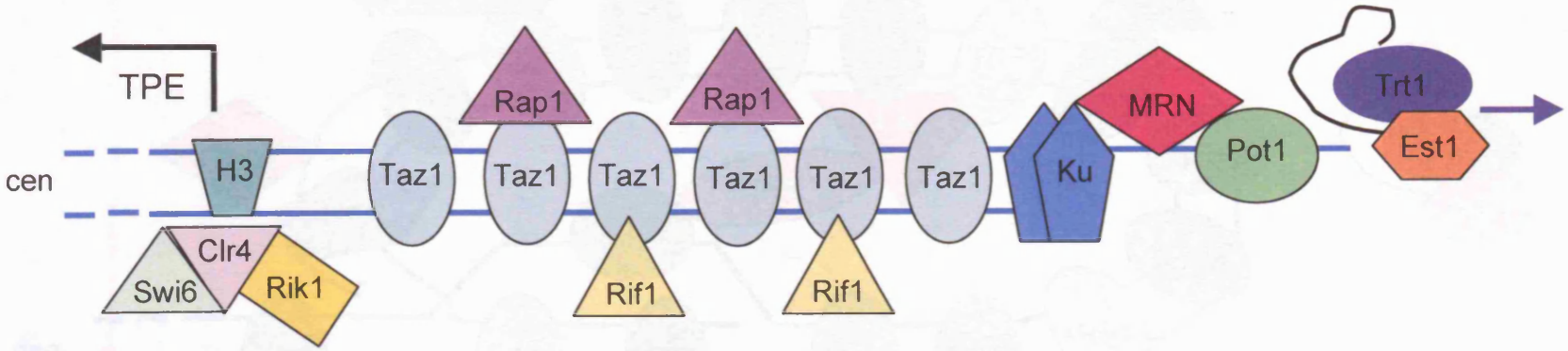
the transcription of the telomeric transposable elements. HP1 mutants show an increase in telomere specific transcripts, probably due to loss of the heterochromatin mediated silencing. This increase in transcript level subsequently causes a lengthening of telomeres (Perrini et al., 2004; Savitsky et al., 2002). The specific functions of HP1 at telomeres can be separated with respect to the requirement of a conserved domain. The chromodomain is essential for silencing of the transposable elements and telomere elongation, but is dispensable for the capping function (Fanti et al., 1998; Perrini et al., 2004).

Another protein located at *Drosophila* telomeres is the HP1/ORC associated protein (HOAP) (Shareef et al., 2001). HOAP is required for the telomere capping function of telomeres (Cenci et al., 2003). Mutations in a class I ubiquitin-conjugating enzyme, UbcD1 also shows a telomere phenotype. The telomere associations observed, however, do not represent fusions as they are resolved during anaphase (Cenci et al., 1997). UbcD1 may play a role in the degradation of telomere proteins that are required for regulated telomere associations, perhaps localising them to nuclear domains.

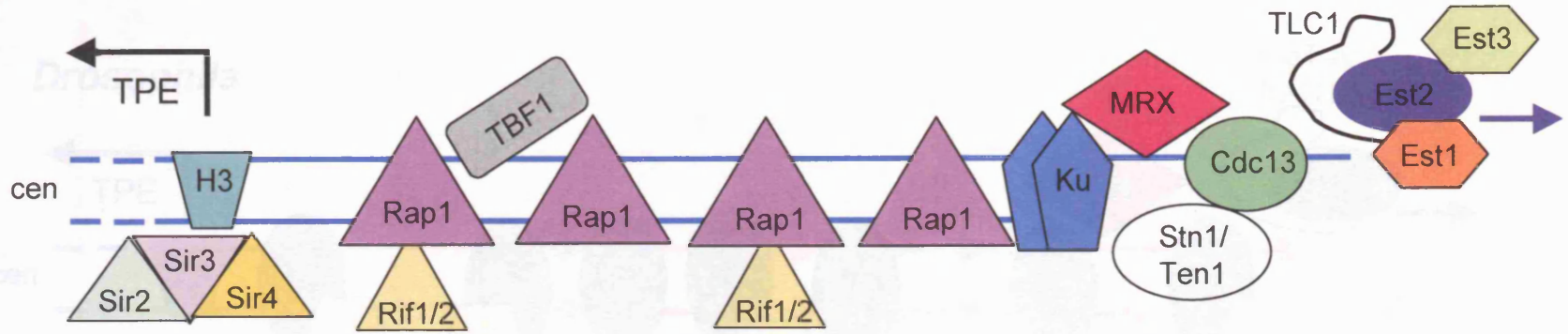
In a similar manner to other eukaryotes with conventional telomeres, proteins involved in DNA repair also seem to be involved in telomere maintenance in *Drosophila.* Mutations in *tef* (the ATM homologue), *Mre11* and *Rad50* cause telomere fusions, anaphase bridges and chromosome breakage, and may be involved in the localisation of HP1 and HOAP to *Drosophila* telomeres (Bi et al., 2004; Ciapponi et al., 2004; Oikemus et al., 2004; Silva et al., 2004). The activity of these enzymes at *Drosophila* telomeres may provide a means of targeting HP1 to chromosome ends. The nuclease activity of the complex may provide a single strand overhang. HP1 has a higher affinity to single-stranded than double-stranded DNA (Perrini et al., 2004) and so may preferentially be recruited to the overhang at chromosome termini. HOAP may also be recruited in an analogous way.

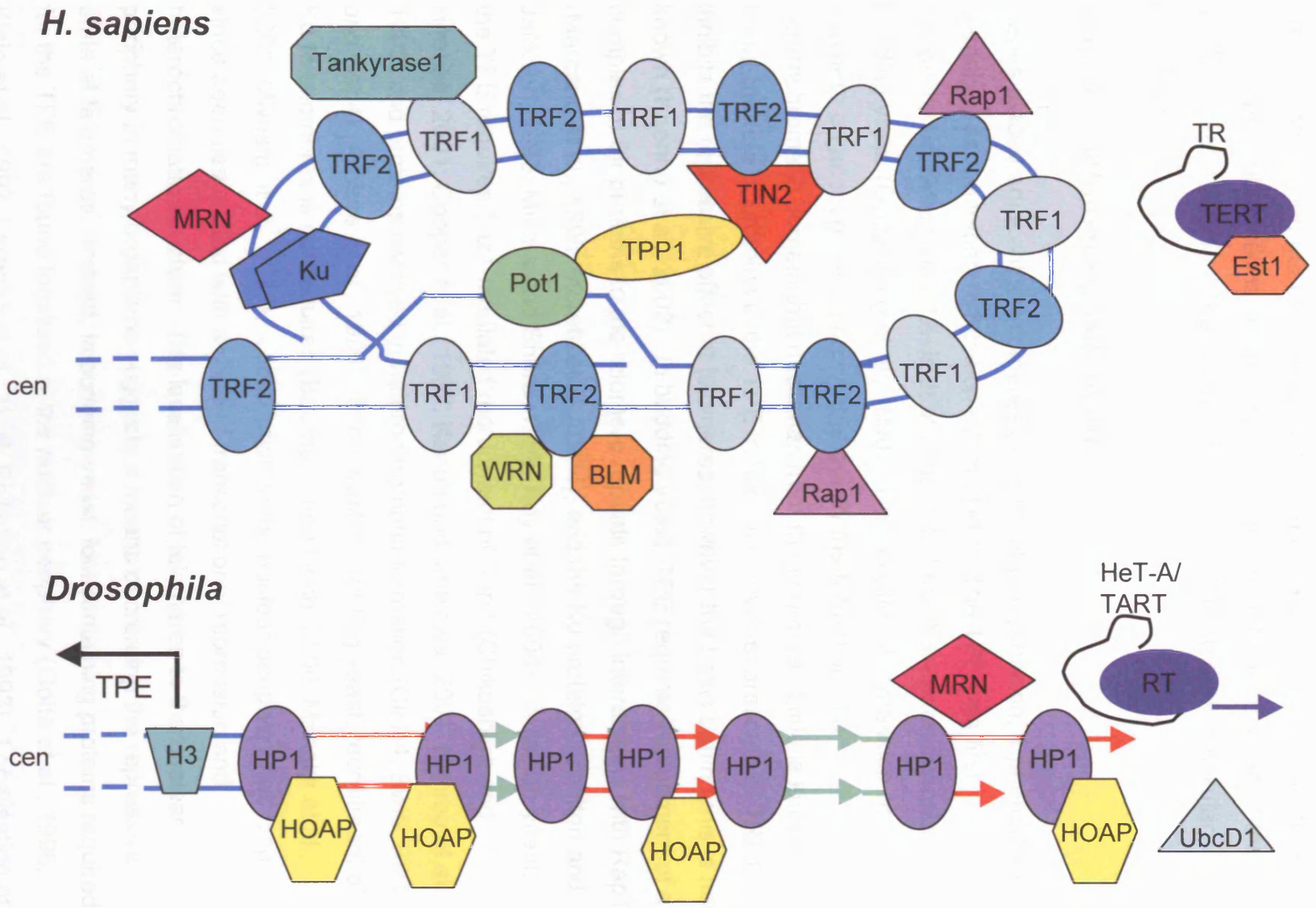
Figure 1.1 Schematic representation of telomeres in Fission yeast, budding yeast, humans and *Drosophila*

S. pombe



S. cerevisiae




H. sapiens
Tankyrase1
TRF2
TRF1
TRF2
TRF1
TRF2
TRF1
Rap1
TRF2
TRF1
TIN2
MRN
Ku
TPP1
Pot1
TRF1
TRF2
cen
TRF2
TRF1
TRF2
TRF1
TRF2
TRF1
WRN
BLM
Rap1
TR
TERT
Est1
Drosophila
HeT-A/
TART
TPE
MRN
RT
cen
H3
HP1
HP1
HP1
HP1
HP1
HP1
HP1
HOAP
HOAP
HOAP
HOAP
UbcD1

1.3 Telomere functions

Telomeres play diverse roles in the maintenance of genome integrity. Many experiments carried out through disrupting telomere structure demonstrate these important functions. In the following section I introduce some of the more important and well characterised functions of telomeres in cellular processes.

1.3.1 The telomere position effect

Genes placed adjacent to telomeres are subject to repression, a phenomenon described as the telomere position effect (TPE). The TPE was first discovered in *Drosophila* (Levis et al., 1985), but has best been described in budding yeast (Gottschling et al., 1990). Transcriptional repression of telomere adjacent genes is brought about by the formation of a heterochromatic domain that nucleates from the telomere. Little is known about the role of proteins in the TPE in humans. Over-expression of TRF1 inhibits the repressive effect at telomeres, however the basis behind this is not known (Koering et al., 2002). In budding yeast, TPE requires recruitment of a complex of Sir proteins to the telomere repeats through interactions with Rap1 (Marcand et al., 1996; Moretti et al., 1994), and the Ku proteins (Boulton and Jackson, 1998; Mishra and Shore, 1999; Roy et al., 2004). In fission yeast, the TPE requires Taz1-mediated recruitment of Rap1 (Chikashige and Hiraoka, 2001; Cooper et al., 1997; Kanoh and Ishikawa, 2001; Nimmo et al., 1998) and proteins involved in heterochromatin formation (Clr1-4, Swi6, Rik1 and Csp4) (Allshire et al., 1995). In contrast to budding yeast, recruitment of Ku to telomeres is not required (Baumann and Cech, 2000; Manolis et al., 2001; Miyoshi et al., 2003). Localisation to the nuclear periphery has long since been associated with a state of transcriptional repression and heterochromatic structure. The localisation of telomeres to the nuclear periphery in many organisms suggests a means of creating the repressive state at telomeres. Indeed, in budding yeast, foci containing proteins required for the TPE are found localised at the nuclear periphery (Gotta et al., 1996; Klein et al., 1992; Laroche et al., 1998; Palladino et al., 1993). Localisation of some telomeres to the nuclear periphery is dependent on Ku, however, following disruption of Ku, some remain associated in a Sir4-dependent

manner (Hediger et al., 2002). The Ku dependence of the telomere associated repression may be due to the Ku dependent localisation of telomeres to the nuclear periphery (Laroche et al., 1998; Taddei et al., 2004). However, Sir-mediated repression can also be maintained out of the context of the nuclear periphery, suggesting localisation to the periphery may not be the determining factor of transcriptional state (Gartenberg et al., 2004). Similarly, tethering DNA to the nuclear periphery does not necessarily cause transcriptional repression (Taddei et al., 2004). Instead, localisation to the nuclear periphery may assist in the formation of repressive loci through concentration of silencing factors, but the actual formation of a repressive domain is likely to be determined by other factors.

1.3.2 Telomeres and the DNA damage response

Perhaps one of the most fundamental roles telomeres play is to differentiate natural chromosome ends from those of chromosomes breaks. As demonstrated by Muller and McClintock in the late 30's, chromosome ends are protected from the potent and potentially detrimental DNA repair mechanisms that exist within the cell (McClintock, 1938; McClintock, 1941; Muller, 1938). A long-standing contradiction in telomere biology is the fact that while telomeres act to prevent a DNA damage response at the natural ends of chromosomes, proteins involved in the DNA damage response are actually present at, and an inherent part of, the telomere structure (Dahlen et al., 1998; Nakamura et al., 2002).

1.3.2.1 The DNA damage response

The cell cycle is tightly regulated to prevent progression in the presence of DNA damage. Failure of a cell to effectively repair damage may cause genome instability and aneuploidy, hallmarks of cancer. Following detection of broken DNA, the G2/M checkpoint pathway delays cell cycle progression and assists in the coordinated repair of the damage. In fission yeast, the ATR homolog, Rad3 is key to initiating both damage and replication checkpoint responses. Specificity of the checkpoint is determined by phosphorylation of the downstream effector kinases, Chk1 for the G2/M damage response and Cds1 for the intra-S-phase replication checkpoint. Phosphorylation of Chk1

ultimately leads to inactivation of the Cdc2-cyclin-B kinase complex, preventing entry into mitosis (Furuya and Carr, 2003; McGowan and Russell, 2004; Rhind and Russell, 1998). The cell cycle delay allows time for processes such as homologous recombination (HR) and non-homologous end joining (NHEJ) to repair the damage, before release of the block and continuation of the cell cycle.

1.3.2.2 Uncapped telomeres

Because of the sensitivity of the DNA damage response, chromosome ends must be protected in order to prevent fusion with other chromosome ends, or indeed genomic breaks. If chromosome ends were to be processed in a similar manner to DSBs, dicentric chromosomes would form which, upon segregation, would lead to breakage and further ends free to be processed; the so called 'breakage-fusion-bridge cycle' (McClintock, 1938). The specialised telomere complex acts to evade this response, which would otherwise lead to genome instability and potentially cancer in higher organisms.

In many experimental systems, loss of the major telomere binding proteins and subsequent disruption of telomere structure leads to chromosome ends being detected and processed and breaks. In mammalian cells, foci known as TIFs (Telomere dysfunction-Induced Foci) are observed at uncapped chromosome ends. These foci contain proteins involved in a DNA damage checkpoint and repair such as γ-H2AX, the MRN complex, 53BP1, ATM and RAD17 (d'Adda di Fagagna et al., 2003; Takai et al., 2003). Formation of TIFs is dependent on activation of the kinase, ATM. However, in the absence of ATM, TIFs form in an ATR dependent manner. Caffeine-mediated inhibition of both of the kinases leads to the abolition of TIF formation. Following activation of the ATM/ATR kinases, activation of the downstream damage pathway ensues; phosphorylation of effector kinases, checkpoint mediated cell cycle delay and apoptosis (Karlseder et al., 1999; van Steensel et al., 1998).

Inhibition of the human telomere binding protein TRF2 leads to uncapping of telomeres, recruitment of DNA damage proteins to chromosome ends,

telomere fusion and activation of an ATM-dependent cell cycle arrest (Karlseder et al., 1999; Smogorzewska et al., 2002; Takai et al., 2003; van Steensel et al., 1998). Despite the fission yeast TFR homologue, Taz1, being the major telomere binding protein in fission yeast, deletion has little effect on growth under optimal conditions (Cooper et al., 1997). The general tolerance observed following loss of a regulated telomere structure after disruption of Taz1 stems from the fact that fission yeast is primarily a G2 organism, without a discernable G1 phase of the cell cycle. Starving cells for nitrogen leads to a G1 arrest. Interestingly, *taz1*Δ mutants arrested in G1 undergo lethal, NHEJ dependent telomere fusions (Ferreira and Cooper, 2001). This is due to the cell cycle regulation of the different repair processes; HR predominates in G2 whereas NHEJ predominates in G1 (Ferreira and Cooper, 2004). In a similar way, chromosome fusions that occur following loss of TRF2 in human cells are formed through NHEJ in a ligase 4-dependent manner (Smogorzewska et al., 2002). The role of Taz1 in protection from end fusion is dependent on it's recruitment of Rap1 to telomeres, but independent of Rif1 recruitment (Miller et al., 2005).

Loss of chromosome end protection by the single strand telomere binding protein, POT1 at human and mouse telomeres (Baumann and Cech, 2001; Hockemeyer et al., 2006), Pot1 at fission yeast telomeres (Baumann and Cech, 2001) and Cdc13 at budding yeast telomeres can be observe following disruption of function. In human cells, loss of POT1 leads a deregulated telomere structure and activation of a DNA damage respose at chromosome ends (Hockemeyer et al., 2005; Veldman et al., 2004; Yang et al., 2005). *pot1*Δ fission yeast cells display immediate loss of all telomeric and subtelomeric DNA followed by senescence and survival only by chromosome circularisation (Baumann and Cech, 2001). Loss of Cdc13 function in budding yeast causes extensive resectioning of the C-strand, resulting in a cell cycle arrest activated by exposure of the single strand overhang (Garvik et al., 1995; Weinert and Hartwell, 1993). It is conceivable that the extensive and immediate telomere loss in fission yeast following disruption of Pot1 may occur through telomere rapid deletion (TRD), promoted by a similar overhang. Intrachromosomal invasion of a long, unprotected single-stranded overhang could result in extensive loss of telomere DNA (Li and Lustig, 1996).

Alternatively, the loss of Pot1 from chromosome ends may lead to extensive degradation by nucleases. A similar role is played by Cdc13 in budding yeast, acting through interactions with Ten1 and Stn1 (Chandra et al., 2001; Grandin et al., 2001; Grandin et al., 1997). Deletion of Cdc13 causes lethal end fusions (Garvik et al., 1995).

Chromosome end uncapping is also observed following loss of a range of other proteins, demonstrating the diversity of proteins involved in telomere maintenance. In fission yeast, loss of the checkpoint kinase, Rad3 (ATR homologue) in combination with either Tel1 (ATM homologue) or MRN causes loss of all telomere DNA and survival through circularisation of each of the three chromosomes (Naito et al., 1998; Nakamura et al., 2002). This defines two pathways that act to prevent loss of telomere DNA in fission yeast; one is dependent on Rad3, the other on Tel1 and the MRN complex. Similarly, loss of the homologues in budding yeast (Mec1 (ATR) and Tel1 (ATM)) causes telomere loss and chromosome end fusions (Craven et al., 2002).

The Ku proteins also play a role in telomere maintenance. In both budding and fission yeast, Ku deletion causes telomere shortening and persistence of the normally S-phase restricted 3' overhang (Baumann and Cech, 2000; Boulton and Jackson, 1996; Gravel et al., 1998; Peterson et al., 2001; Polotnianka et al., 1998; Stellwagen et al., 2003; Tomita et al., 2003). Combining mutations of Ku with those in the telomerase catalytic subunit causes accelerated senescence in both fission and budding yeast (Baumann and Cech, 2000; Gravel et al., 1998; Nugent et al., 1998). However, whereas this is lethal in budding yeast with no survivor populations arising, in fission yeast, survivor populations arise. This may reflect a difference in the survival mode employed by the different types of yeast in the absence of telomerase, with budding yeast maintaining linear chromosomes and fission yeast, chromosomes as circles. In mice, the role of Ku at telomeres is contradictory. In one report, Ku deletion leads to telomere fusion without loss of telomere repeats suggesting a role of Ku in telomere capping (Samper et al., 2000). The other report, supported by data in yeast, suggests a role for Ku in telomere length maintenance; the fusions only occurring following loss of telomere DNA (d'Adda di Fagagna et al., 2001). In human cells, Ku is

essential making analysis difficult (Li et al., 2002). However, reducing Ku levels causes telomere loss and end fusion demonstrating its role in telomere protection (Jaco et al., 2004; Myung et al., 2004).

Loss of chromosome end capping is also observed following loss of the telomere repeats themselves. In cells lacking telomerase function, either through loss of components of the enzyme itself or accessory factors required for its function, telomeres shorten as cells divide (see Chapter 1.3.3 for more details). Primary human cell lines lacking telomerase display telomere shortening accompanied by telomere fusions and increased levels of apoptosis (Harley et al., 1990; Hastie et al., 1990). Similarly, disruption of telomerase in other organisms and subsequent telomere attrition leads to an irreversible cell cycle arrest caused by lethal end fusions. Telomerase knockout mice do not exhibit a phenotype until late generations, as they start off with such long telomeres that critically short lengths are not observed until the 4^{th} to 6^{th} generation. Embryonic fibroblasts from G4-G6 mice show increased frequency of aneuploid cells and end-to-end fusions due to the increased frequency of chromosome ends lacking telomere signal (Blasco et al., 1997; Hande et al., 1999; Lee et al., 1998; Rudolph et al., 1999). Consistent with these observations, late generations mice show a range of defects. An increase in apoptosis and decrease in cell proliferation was observed in later generation mice, particularly in highly proliferative tissues (Lee et al., 1998). G6 mice are sterile, stemming from germ cell depletion in both the male and female (Hemann et al., 2001; Herrera et al., 2000; Lee et al., 1998). Rescue of defects can be observed upon reconstitution of telomerase activity and subsequent lengthening of telomeres (Hemann et al., 2001; Samper et al., 2001). Similarly, loss of telomerase function in budding and fission yeast leads to loss of telomere DNA, chromosome end uncapping, chromosome end fusions and senescence (Beernink et al., 2003; Lendvay et al., 1996; Lingner et al., 1997; Lundblad and Szostak, 1989; Nakamura et al., 1998; Singer and Gottschling, 1994). In these situations, however, survivor populations may arise, as discussed in Chapter 1.3.4.

It is interesting to note that in *Drosophila,* loss of terminal telomere repeats does not necessarily lead to loss of the protective capping. Chromosomes

lacking the terminal transposons have been observed and can be transferred stably to subsequent generations (Biessmann et al., 1990; Levis, 1989; Mason et al., 1984). This observation is contradictory to the initial observations made in *Drosophila* that led to the concept of the telomere (Muller, 1938). Suggestions have been made that the discrepancies may be due to the genetic background or method of inducing the break. Indeed the terminal deletion phenotypes are often observed in flies carrying mutations in the *mu-2* locus which is thought to affect the processing of DSBs (Biessmann et al., 1990; Mason et al., 1997; Mason et al., 1984). Despite the ability to maintain stable chromosomes lacking terminal repeats, binding of the telomere associated proteins to the terminal telomere structure seems to be a prerequisite for chromosome stability in Drosophila, irrespective of terminal sequence (Cenci et al., 2003; Fanti et al., 1998; Perrini et al., 2004). Therefore, at least in Drosophila, the recruitment of proteins at chromosome termini and perhaps the structure created by these proteins, irrespective of DNA sequence, is the important contributory factor to providing protection at chromosome ends.

1.3.2.3Telomeres and DNA repair

It is also becoming more apparent that some proteins identified due to their involvement in telomere maintenance also play a secondary role in the DNA damage response. The human telomere binding protein TRF2 is phosphorylated in an ATM dependent manner in an early response to DNA damage and transiently migrates to sites of damage following high levels of laser irradiation (Bradshaw et al., 2005; Tanaka et al., 2005).

It has also been observed that *taz1*Δ strains are sensitive to agents that induce DNA double strand breaks, suggesting a role for the protein or functional telomeres in surviving DNA damage (Miller and Cooper, 2003). While the sensitivity of a *taz1*Δ mutant to damage is mild, sensitivity is greatly exacerbated in combination with loss of either *bub1*, *mph1* or *cds1*, loss of any of which does not confer sensitivity in the presence of Taz1 (Miller and Cooper, 2003). Evidence in other organisms also suggests a role of telomeres or associated proteins in repair of DNA damage. Mice lacking telomerase display a general sensitivity to alkylating agents and γ-irradiation,

but only in late generations when telomeres are shortened (Gonzalez-Suarez et al., 2003; Goytisolo et al., 2000; Wong et al., 2000). Telomerase negative human cell lines are sensitive to ionising radiation due to dysfunctional telomere structure, rather than length (Rubio et al., 2002).

A further role of telomeres in the repair of DNA damage has been described in budding yeast. Telomere clustering and anchoring to the nuclear periphery occurs via an association with the nuclear pore complex (NPC) (Galy et al., 2000; Therizols et al., 2006). While this association does not affect the general DNA repair efficiency, it is required to promote efficient repair of DSBs created in the subtelomeric regions. The ability to repair subtelomeric DSBs depends on the tethering to the nuclear periphery, and perhaps clustering of telomeres, but is independent of the ability to silence subtelomeric transcription, suggesting the heterochromatic nature of the telomere is not required for efficient repair of subtelomeric DSBs. A mutant lacking Esc1 shows disrupted nuclear tethering and subtelomeric repair, but telomeric silencing remains intact (Therizols et al., 2006). The basis for this is not known, but it could be the concentration of repair proteins to one domain within the nucleus. Alternatively, the clustering of telomeres may promote efficient repair of these repetitive sequences by HR. It was previously shown that the efficiency of repair by NHEJ in chromosomes lacking a homologous partner declines towards the telomere region. In these regions, repair by other mechanisms such as HR and BIR increase, particularly in regions lacking essential genes (Ricchetti et al., 2003). Organisation within the nucleus may promote the type of repair that is most favourable to a particular region of the genome, creating nuclear subcompartments, with specific repair proteins focused within the domain.

Chromosome healing by telomerase has been observed in organisms including budding yeast (Kramer and Haber, 1993; Putnam et al., 2004), mouse cell lines (Sprung et al., 1999) and human cell lines (Fouladi et al., 2000; Sabatier et al., 2005). Chromosome breaks are stabilised through the addition of *de novo* telomere repeats to the ends. Telomere healing is likely to play an important part in the progression of tumorigenesis. During tumour formation, chromosomes undergo many rearrangements such as terminal

deletions and translocations. The stabilisation of chromosome ends is an important step in the malignant transformation. Indeed, cancer cells with terminal deletions capped by telomeres are observed (Kawai et al., 2002).

1.3.3 Telomerase and the end replication problem

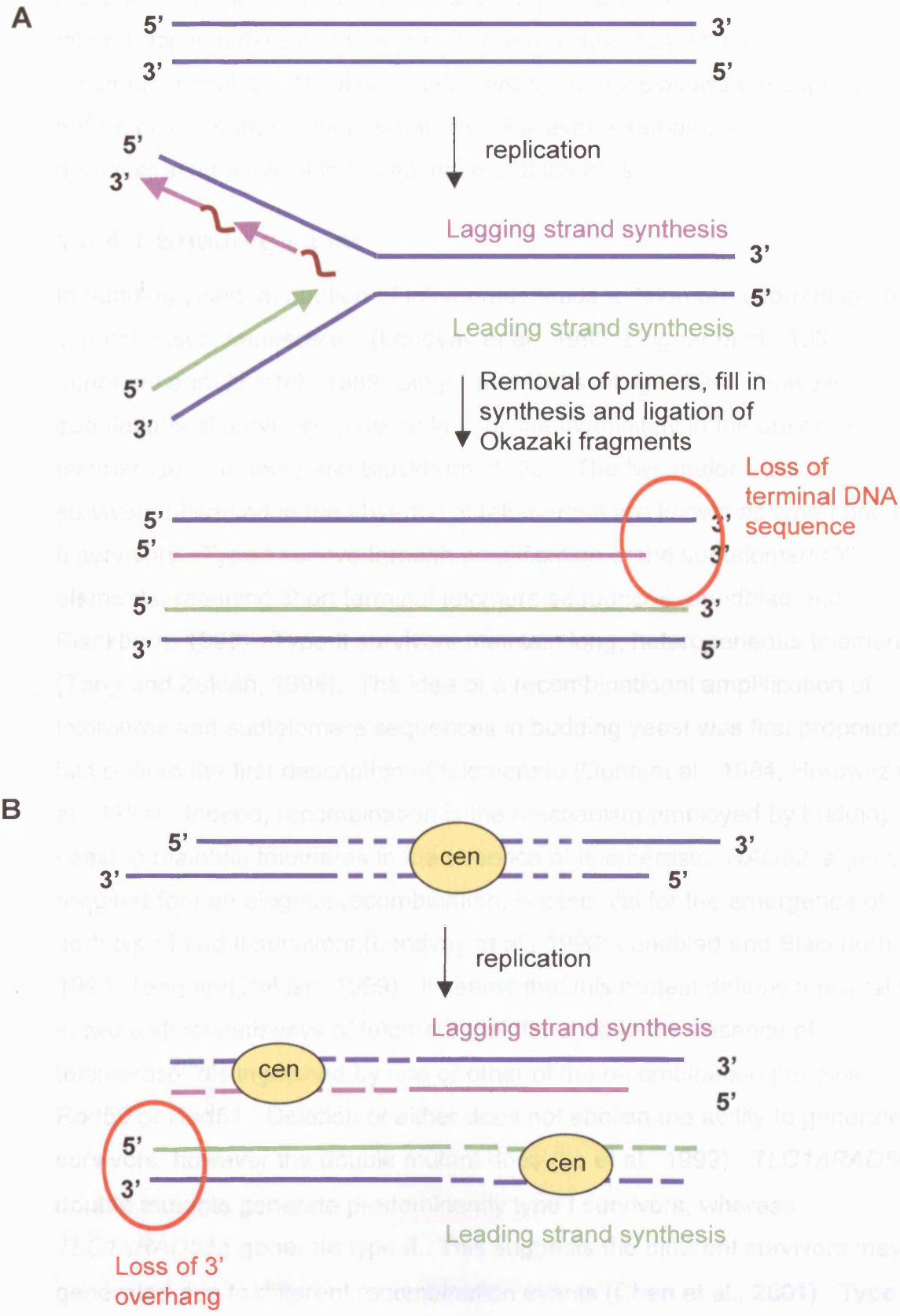
Conventional DNA replication machinery is not able to fully replicate chromosome ends (Watson, 1972). DNA polymerase can only replicate in a 5' to 3' direction. Replication of the lagging strand requires RNA primers to initiate replication, synthesising the new DNA as short Okazaki fragments. Following replication, the RNA primers are removed and replaced by fill in synthesis of DNA and ligation of the fragments. However, the primer from the terminal Okazaki fragment is not replaced, causing loss of DNA from the 5' end and a 3' overhang (Figure 1.2 A). The discovery that the chromosome termini are capped with specialised structures, with a 3' overhang being an integral part of the structure created an additional problem (Lingner et al., 1995). Replication of the leading strand would continue to the end of the molecule using conventional polymerases, however because of the lack of template, the 3' overhang structure would be lost, creating a blunt ended molecule. Therefore, if telomeres were to be replicated by conventional polymerases, both DNA sequences and the terminal structure would be lost (Figure 1.2 B).

To counteract these problems, a ribonucleoprotein, telomerase exists and acts by adding telomere-specific repeats to the chromosome ends. Disruption of telomerase leads to telomere shortening with subsequent cell divisions and ultimately cell death in most cases. This is due to the occurrence of lethal fusions as the chromosome ends are effectively uncapped when the telomeres become critically short, and are recognised and processed as DNA breaks. Telomerase acts preferentially on a subset of telomeres in each cell cycle, preferentially elongating the shorter telomeres (Teixeira et al., 2004). Telomerase is not active in most somatic human cells. This is thought to act as a mechanism to guard against cancer by limiting the proliferative capacity of cells.

Figure 1.2 The end replication problem

(A) Lagging strand synthesis requires an RNA primer. Removal of the primer following DNA synthesis leads to loss of the terminal sequence and a 3' overhang.

(B) Replication of a molecule with a 3' overhang leads to loss of the 3' overhang DNA and structure.


A
5'
3'
3'
5'
replication
5'
3'
Lagging strand synthesis
3'
5'
Leading strand synthesis
5'
3'
Removal of primers, fill in synthesis and ligation of Okazaki fragments
Loss of terminal DNA sequence
5'
3'
5'
3'
5'
3'
3'
5'
B
5'
3'
cen
3'
5'
replication
Lagging strand synthesis
cen
3'
5'
5'
3'
cen
Leading strand synthesis
Loss of 3' overhang

1.3.4 Survival without telomerase

A broadening mode of survival is becoming apparent in the absence of telomerase in different organisms. In the majority of these cases, recombinational amplification of telomere sequences allows the capping nature of telomeres to be maintained. However, examples exist demonstrating survival in the absence of telomeres.

1.3.4.1 Budding yeast

In budding yeast, disruption of telomerase leads to telomere shortening and, in most cases, senescence (Lendvay et al., 1996; Lingner et al., 1997; Lundblad and Szostak, 1989; Singer and Gottschling, 1994). However, populations of survivors arise, able to divide indefinitely in the absence of telomerase (Lundblad and Blackburn, 1993). The two major types of survivors observed in the absence of telomerase are known as type I and type II survivors. Type I survive through amplification of the subtelomeric Y' elements, retaining short terminal telomere sequences (Lundblad and Blackburn, 1993). Type II survivors maintain long, heterogeneous telomeres (Teng and Zakian, 1999). The idea of a recombinational amplification of telomeres and subtelomere sequences in budding yeast was first proposed just prior to the first description of telomerase (Dunn et al., 1984; Horowitz et al., 1984). Indeed, recombination is the mechanism employed by budding yeast to maintain telomeres in the absence of telomerase. *RAD52*, a gene required for homologous recombination, is essential for the emergence of both type I and II survivors (Lendvay et al., 1996; Lundblad and Blackburn, 1993; Teng and Zakian, 1999). It seems that this protein defines a pivotal role in two distinct pathways of telomere maintenance in the absence of telomerase, distinguished by one or other of the recombination proteins, Rad50 or Rad51. Deletion of either does not abolish the ability to generate survivors, however the double mutant does (Le et al., 1999). *TLC1ΔRAD50Δ* double mutants generate predominantly type I survivors, whereas *TLC1ΔRAD51Δ* generate type II. This suggests the different survivors may be generated due to different recombination events (Chen et al., 2001). Type II survivors also require Tel1 and Mec1 (Tsai et al., 2002). Possibly the emergence of this type of survivor requires a cell cycle arrest.

In the absence of Sgs1, the RecQ helicase homologue, telomerase deficient strains senesce more rapidly than in the presence, possibly due to the inability to resolve secondary DNA structures, leading to inefficient replication and therefore more rapid loss of telomere DNA (Cohen and Sinclair, 2001; Huang et al., 2001). Sgs1 plays a role in the Rad50-dependent survivor pathway and is required for the emergence of type II survivors (Huang et al., 2001; Johnson et al., 2001). This supports a proposed role of break-induced-replication (BIR) in maintenance of telomere DNA in the absence of telomerase. BIR intermediates resemble stalled replication forks, a structure Sgs1 localises to (Frei and Gasser, 2000; Haber, 1999). Sgs1 has also been shown to prevent recombination in the subtelomeric Y' elements, leaving only the type I survival mode (Watt et al., 1996). Sgs1 helicase may also provide a positive action on the emergence of type II survivors rather than just preventing recombination of Y' elements. The helicase function may act upon the telomere repeats, allowing a more open conformation, promoting recombination at the telomere regions.

There may not always be such a clear-cut division between the two types of survival mechanism in the presence of both recombination-based pathways. Type I survivors, labelled so due to their lack of extensive telomere repeat sequence, may not always show extensive amplification of Y' elements. Similarly, type II survivors may have telomeres that are still short. Type I survivors will often convert to the faster growing type II survivors, amplifying telomere tracts, but retaining the amplified Y' elements (Teng and Zakian, 1999). This suggests that in the presence of both RAD50 and RAD51, both pathways may be employed, perhaps to different extents, to maintain telomere DNA.

Type I and II survivors are not stable and cultures may go through repeated rounds of recombinational telomere elongation. It is particularly notable in type II survivors where telomeres continue to shorten until they reach a critically short length and are lengthened again following a further short period of senescence (Teng et al., 2000). While recombination clearly acts at telomerase negative telomeres and plays an important part in their

maintenance, it may be that it is a transient action, acting only to elongate telomeres as they become critically short.

EXO1, a flap endonuclease (Tran et al., 2002) plays roles in generating a single strand overhang through resection of DSBs (Fiorentini et al., 1997) and uncapped telomeres (Lydall, 2003; Maringele and Lydall, 2002). Generation of type I and II telomerase negative strains requires EXO1 (Maringele and Lydall, 2004a). It is likely to be required to create the single strand DNA that will invade the homologous region to initiate the recombination process. In the absence of EXO1, recombination pathways and telomerase, survival occurs through the formation of large palindromes at chromosome ends (Maringele and Lydall, 2004b). The chromosomes in these so-called PAL survivors are highly rearranged and many display a significantly reduced or even lack all telomere signal, demonstrating an alternative mechanism to maintain linear chromosomes in the absence of telomere repeats.

1.3.4.2 Human

In immortalised human cell lines, telomere maintenance in the absence of telomerase occurs by a mechanism known as ALT (Alternative Lengthening of Telomeres) (Bryan et al., 1995; Dunham et al., 2000). Approximately 5-10% of tumours use an ALT pathway of telomere maintenance as an alternative to telomerase (Bryan et al., 1997; Colgin and Reddel, 1999). While the molecular mechanism of ALT is not known, it is also thought to be recombination-based, as suggested by the variable telomere lengths. The model is further supported by an experiment whereby integration of a tagged plasmid into the telomere region was shown to spread to other chromosomes with an increase in population doublings. This spreading occurs only in ALT cell lines, not telomerase positive or mortal lines, suggesting recombination is a feature specific to the survival mechanism (Dunham et al., 2000). Survival by the ALT mechanism is identified by a set of characteristics found in nearly all immortal lines lacking telomerase. ALT telomeres, like type II survivors in budding yeast, are very heterogeneous in length, ranging from less than 3kb to greater than 50kb, even within individual cells (Bryan et al., 1995; Grobelny et al., 2000; Lansdorp et al., 1997; Murnane et al., 1994; Perrem et al., 2001). They also display a high rate of post-replicative sister chromatid exchange

(Bailey et al., 2004; Bechter et al., 2004; Londono-Vallejo et al., 2004). Whether this is involved in telomere maintenance or just a consequence of the ALT mechanism is not known. It is conceivable that exchanges of DNA from chromosome arms containing long stretches of telomeres, to those containing shorter stretches, could provide a means of lengthening some chromatids (and shorten others) before mitosis without the need to synthesise new telomere DNA. Alternatively, the greater presence of proteins involved in mitotic HR at telomeres for the ALT mechanism may generate sister chromatid exchange as a byproduct.

Another characteristic of ALT cell lines is the existence of specialised nuclear structures. Almost all human ALT cell lines have associated nuclear structures called APBs (ALT- associated Promyelocytic leukaemia Bodies), although they are seen in only a portion of the cells within a population. This may be due to a cell cycle regulation of the structures and the stage of the cell cycle at which the cells are observed (Yeager et al., 1999). PML bodies are involved in an array of cellular processes including regulation of the cell cycle, senescence, apoptosis and chromatin modification. They are thought to assist in these processes by sequestering and releasing proteins involved in these processes, bringing them to sites of action, and facilitating interactions (Hodges et al., 1998). APBs contain telomere associated proteins, including the TRF proteins, proteins involved in DNA repair and also extrachromosomal telomeric DNA (Nabetani et al., 2004; Silverman et al., 2004; Wu et al., 2003; Wu et al., 2000; Yeager et al., 1999; Zhu et al., 2000). APBs may assist in the process of maintaining telomeres through recombination in the absence of telomerase. Alternatively, they may be involved in removing extrachromosomal DNA from chromosome ends created in the process of amplifying telomeres by the ALT mechanism.

Despite these characteristics being observed in the majority of immortal cell lines lacking telomerase, exceptions have been observed. An immortal cell line derived from ALT cells lacking telomerase has been described lacking many of the characteristic features of the ALT mechanism. While this cell line retains high levels of sister chromatid exchange, telomere lengths are short and homogeneous in size. In addition, the cells lack APBs and

extrachromasomal telomere DNA (Cerone et al., 2005). Other lines have been described lacking APBs (Fasching et al., 2005). A further cell line has been described lacking classical features of the ALT mechanism, such as APBs, but with characteristics perhaps more reminiscent of type I survivors of budding yeast (Marciniak et al., 2005). These telomerase negative cells have short stretches of the telomere repeat unit interspersed with tandem repeats of SV40 sequence. Furthermore, the telomere containing sequences in this cell line are transcribed, probably from the integrated SV40 promotor sequence. Interestingly, this cell line lacks the Sgs1 homolog, WRN, which in budding yeast is required for emergence of type II survivors (Marciniak et al., 2005). These findings suggest that while the classically described alternative mechanism for lengthening telomeres in the absence of telomerase may predominate, other mechanisms also exist.

1.3.4.3 Fission yeast

In fission yeast, survival without telomerase occurs primarily by a different mechanism made possible by the small number of chromosomes. Each of the three chromosomes undergo intramolecular fusion, forming individual circles having lost all telomeric and most of the subtelomeric DNA (Nakamura et al., 1998). In other organisms, the greater number of chromosomes means survival by this mechanism is impossible. Circular survivors are also seen in other fission yeast mutants defective for proteins involved in telomere maintenance, including *pot1*Δ and strains lacking both of the checkpoint kinases, Rad3 and Tel1 (Baumann and Cech, 2001; Naito et al., 1998). Survival by chromosome circularisation is, surprisingly, not dependent on the NHEJ activities of Ku or Rad22-dependent HR (Baumann and Cech, 2000; Nakamura et al., 2002). This suggests that the mechanisms involved in end joining at chromosome ends may be different from those acting at DNA double strand breaks. Interestingly, the fusions observed in *taz1*Δ telomeres are Ku-dependent, demonstrating a fundamental difference between chromosome ends that are uncapped through loss of a telomere protein, but perhaps retaining some telomeric structure through binding of other telomere associated proteins, and loss of all telomere associated DNA and binding sites for the associated proteins.

A less common recombination-based method of amplifying telomeric DNA is also observed in fission yeast lacking telomerase, maintaining linear chromosomes with telomeres of a heterogeneous length (Nakamura et al., 1998). Survival by this method is only observed in a wild type background when cultured in liquid following disruption of telomerase. The faster growing nature of the linear survivors means they can outgrow the more common circular survivors. The linear survivors that do emerge are not stable, often showing continued telomere shortening and the eventual emergence of circular survivors (Nakamura et al., 1998). Repression of the recombination-based survival occurs due to the presence of the telomere binding protein, Taz1. Taz1 prevents recombination between telomeres (Miller et al., 2006; Nakamura et al., 1998). In the absence of both telomerase and Taz1, cells maintain stable linear chromosomes capped by long heterogeneous telomere repeats (Nakamura et al., 1998).

1.3.4.4 Telomere recombination methods

Recombination based modes of telomere maintenance in the absence of telomerase may utilise a number of possible template DNAs. Various similar models have been proposed around the idea of an initiating strand invasion followed by BIR or gene conversion. Interchromasomal strand exchange is perhaps the most obvious model and a significant possibility (Figure 1.3 A). Indeed, spreading of a tagged sequence associated with a specific telomere to other telomeres has been observed in human and yeast cells (Dunham et al., 2000; Topcu et al., 2005). Another model utilises the formation of the telomere specific structures, or T-Loops, discussed earlier in the chapter. Intrachromosomal invasion of the 3' overhang into a more centromeric region within the telomere, followed by strand extension, using the invaded region as a template, coupled with lagging strand synthesis could allow significant lengthening of the telomere sequence (Figure 1.3 B). However, this model may also cause extensive telomere shortening, or telomere rapid deletion (TRD) a phenomenon first described in yeast (Li and Lustig, 1996). The generation of the extrachromosomal telomeric DNA created by TRD, be it as a circular or linear molecule, may itself be directly involved in the ALT

mechanism. Extrachromosomal telomere DNAs are commonly observed in ALT cell lines and yeast survivors maintaining telomere DNA by recombination (Cesare and Griffith, 2004; Groff-Vindman et al., 2005; Wang et al., 2004). A model known as the known as the 'rolling circle' model of telomere replication was first described in the yeast *Kluyveromyces lactis* (Natarajan and McEachern, 2002). Again, this model begins with strand invasion of the template DNA molecule (this time extrachromosomal circular telomeric DNA) by the 3' overhang of the chromosomal telomere, followed by branch migration. As the chromosome end is extended, the circle is rolled, allowing extensive telomere lengthening (Figure 1.3 C). A variation on this method exists, known as the 'roll and spread', and is perhaps more common than the rolling circle method. In this case, a single telomere is extended using a telomere circle as the template. This long telomere is then spread to other chromosome arms by gene conversion events (Natarajan and McEachern, 2002).

While telomeric circles have yet to be identified in *Sacharomyces cerevisiae* telomerase negative survivors, the telomere patterns in type II survivors are consistent with the 'roll and spread' model. The extensive elongation of telomeres without the presence of obvious intermediate lengths, together with the homogenous structure of telomeres within a given clone (despite heterogeneity between clones) supports the model (Teng et al., 2000). Subtelomeric Y' circles have been observed in wild type cells (Horowitz and Haber, 1985). Conceivably, these circles may provide a means of amplification of repeats in type I survivors. Indeed, the rate of excision of circles containing Y' DNA increases as survivor populations emerge. However, the populations that have been observed to arise with such circles are type II survivors, and so the mechanism can not be directly attributed to the excision of these Y' circles. Instead it is likely to reflect the instability of the terminal sequences as they undergo rearrangements during the emergence of survivor populations (Lin et al., 2005). Whether the circles are elevated in and contribute to type I survival by a rolling circle mechanism needs to be addressed further.

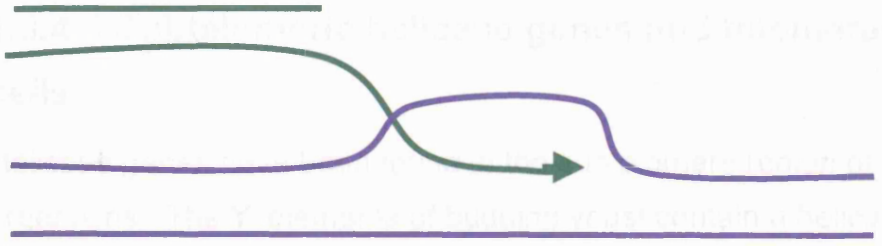
Figure 1.3 Possible templates for telomerase negative telomere elongation

(A) Interchromosomal strand invasion. The 3' single strand overhang from one telomere invades the centromere proximal region of a different telomere. The invading telomere is extended using the other telomere as a template.

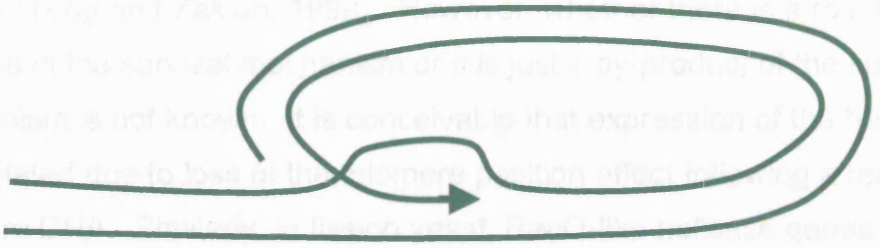
(B) Intrachromosomal strand invasion. The 3' single strand overhang loops back and invades the centromere proximal region of itself. The telomere is extended using itself as a template.

(C) Extrachromosomal strand invasion. The 3'single strand overhang invades extrachromosomal telomere circles. The telomere is extended using the circular DNA as a template. Synthesis may continue round the circle extending a long telomere (rolling circle mechanism). The elongated telomere may itself then be used as a template to elongate other telomeres (roll and spread mechanism)

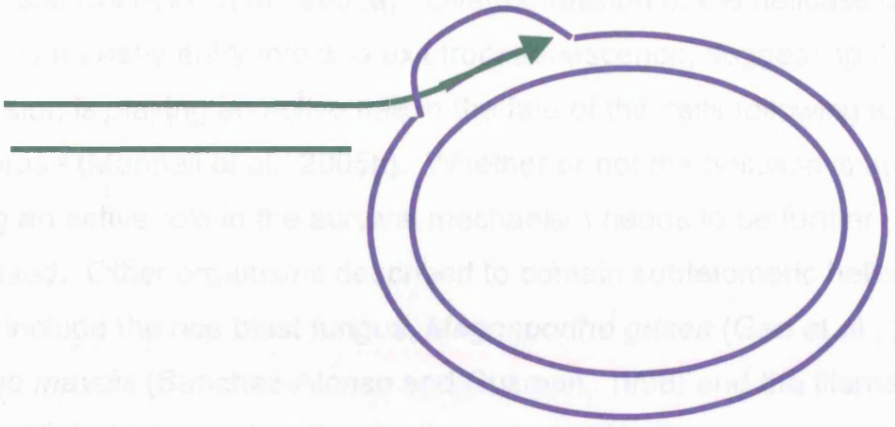
A Interchromosomal



B Intrachromosomal



C extrachromosomal



Interestingly, telomere maintenance by the rolling circle mechanism has been proven. In the yeast *Candida parapsilosis*, linear mitochondrial DNA molecules are maintained with terminal tandem repeats in the absence of a telomerase proficient to lengthen the mitochondrial telomere repeats (Nosek et al., 1995). It has been shown that these telomeres are maintained by a rolling circle mechanism of telomere elongation (Nosek et al., 2005).

1.3.4.5 Subtelomeric helicase genes and telomerase deficient cells

Helicase genes have been found in the subtelomere region of a variety of organisms. The Y' elements of budding yeast contain a helicase, Y'-Help1, transcription of which is induced in type I telomerase negative survivors (Yamada et al., 1998). While the Y' element is transcribed in telomerase negative survivors, a cDNA mediated, transposon-style movement is not likely to be the mechanism for end maintenance as the frequency of movement is too low (Teng and Zakian, 1999). However, whether there is a role for the helicase in the survival mechanism or it is just a by-product of the survival mechanism is not known. It is conceivable that expression of the helicases is upregulated due to loss of the telomere position effect following a reduction in telomere DNA. Similarly, in fission yeast, RecQ-like helicase genes have been identified in four of the six subtelomeric regions (Mandell et al., 2005a; Mandell et al., 2005b). Upregulation of the helicase is observed as cells go through crisis following loss of telomerase and it is the only gene with significantly altered expression at a late stage of recovery from loss of telomerase (Mandell et al., 2005a). Overexpression of the helicase domain promotes an early entry into and exit from senescence, suggesting its expression is playing an active role in the fate of the cells following loss of telomerase (Mandell et al., 2005b). Whether or not the helicase is actually playing an active role in the survival mechanism needs to be further addressed. Other organisms described to contain subtelomeric helicase genes include the rice blast fungus, *Magnaporthe grisea* (Gao et al., 2002), *Ustilago maydis* (Sanchez-Alonso and Guzman, 1998) and the filamentous fungus, *Metarhizium anisopliae* (Inglis et al., 2005). The genes may provide a means for the organisms to adapt when faced with selective pressures. Upon disruption of telomerase and the subsequent telomere erosion in budding

yeast, activation of the Ty1 transposable element is observed (Scholes et al., 2003). The ty1 element has been shown to be involved in the mobilization of the subtelomeric Y' elements in type I telomerase deficient survivors (Maxwell et al., 2004). A common feature of transposons is their ability to respond to stress (Capy et al., 2000).

1.3.5 Telomeres and meiosis

Telomere dynamics during meiosis are very striking, but at present the mechanisms behind the dynamics and proposed roles are largely speculative. More than a century ago, cytological analysis revealed that during meiosis, chromosomes cluster into an arrangement called the 'bouquet structure'. The point at which the chromosomes cluster has since been shown to be the telomeres. The role for such a structure is largely unknown, but suggestions that it promotes homologous pairing and recombination are attractive.

During meiotic interphase in fission yeast, chromosomes are associated with the spindle pole body (SPB) via the centromere. Telomeres remain at the nuclear periphery. Upon induction of meiosis, during the premeiotic phase, there is a switch; centromeres dissociate from the SPB and telomeres associate (Chikashige et al., 1994; Chikashige et al., 1997). During meiosis, the nucleus undergoes a movement, back and forth within the cell, known as the horsetail movement. This movement is lead by telomeres (Chikashige et al., 1994).

In fission yeast, meiosis lacking Taz1 displays disrupted telomere clustering with the SPB. Meiotic recombination is reduced and missegregation of chromosomes is often observed. The resulting asci are usually aberrant with fewer than the typical four spores observed in healthy asci (Cooper et al., 1998; Nimmo et al., 1998). Part of the defect in *taz1*Δ strains undergoing meiosis can be attributed to fusion events occurring during the G1 arrest, a stage that is a prerequisite for meiosis. However, the meiotic defect cannot solely be attributed to end fusions, as blocking end fusions by deletion of *lig4* does not fully suppress the defects of *taz1*Δ meiosis. Binding of Rap1 to telomeres is also required for efficient progression through meiosis in both budding and fission yeast (Alexander and Zakian, 2003; Chikashige and

Hiraoka, 2001). The *taz1*Δ defect in fission yeast is largely suppressed through fusion of Rap1 with the DNA binding domain of Taz1, suggesting that Taz1 acts through the recruitment of Rap1 to telomeres (Chikashige and Hiraoka, 2001).

Meiosis lacking Rik1, a protein involved in heterochromatin formation, shows disrupted meiotic telomere clustering but lacks the telomere end fusions from the G1 stage. *rik1*Δ meiosis displays an intermediate spore viability between wild type and *taz1*Δ strains. Rik1 acts to recruit the methyltransferase, Clr4 to the telomeres to establish a heterochromatin domain. Clr4 is also required for efficient meiosis (Tuzon et al., 2004). The requirement of telomeric heterochromatin formation seems to be a common requirement for proficient meiosis. The budding yeast methyltransferase, Set1 is also required for meiotic telomere clustering (Trelles-Sticken et al., 2005). However, the telomeric heterochromatin requirements for meiosis are not completely comparable with the requirements for all telomere functions. In fission yeast, Swi6 is required for the heterochromatin mediated TPE but is dispensable for meiosis (Tuzon et al., 2004). Similarly, in budding yeast, meiosis does not require Sir3, a protein required for telomere silencing (Trelles-Sticken et al., 2003).

Defective meiosis is observed following loss of telomere DNA in budding yeast and mice (Hemann et al., 2001; Liu et al., 2002a; Liu et al., 2002b; Maddar et al., 2001). Fission yeast strains lacking telomeres, surviving with circular chromosomes, show severe meiotic defects (Naito et al., 1998; Nakamura et al., 1998). Whether these defects are due to a lack of telomere sequence, the topology of the chromosomes, or a combination of the two is unknown. However, two types of circular survivor with different meiotic behaviours have been identified (Sadaie et al., 2003). Type A derivatives show high frequencies of subtelomeric DNA associating with SPB in meiosis (>50% cells show association), type B derivatives show low association (<20%) and type AB show an intermediate association. The association is Taz1 dependent and correlates with the ability to undergo pairing of homologous chromosomes and the amount of STE retained prior to chromosome circularisation (Sadaie et al., 2003). Therefore clustering of

telomeres is not dependent on the presence of the telomere repeat sequences, but rather the structures created by them, such as heterochromatin. However, no report was made as to the effect the ability to pair/not pair had on the outcome of meiosis in these strains. A strong possibility is that the defect may be brought about, at least in part, by circular chromosomes undergoing homologous recombination during meiosis, resulting in dicentric chromosome circles.

1.4 Telomeres and human disease

Because of the complexity of the telomere structure, and particularly the involvement of a diverse range of proteins involved in maintenance of genome integrity also having roles at telomeres, the range of diseases showing a telomere phenotype may not be a surprise. Some of the diseases are likely to have a telomere phenotype as a by-product, with it perhaps contributing to the development or prognosis of the disease. Ataxia telangiectasia is caused by mutations in the genes encoding the checkpoint kinases, ataxia telangiectasia mutated (ATM) and ataxia telengiectasia related (ATR). These proteins associate with telomeres and patients with the disorders display shortened telomeres. Similarly, patients with Fanconi's anaemia also display telomere shortening (Leteurtre et al., 1999). The human RecQ helicases, WRN and BLM have emerging roles at telomeres. These proteins are mutated in Werner's and Bloom's syndromes, causing premature aging and cancer predisposition (Franchitto and Pichierri, 2002). However, with other diseases the role of dysfunctional telomeres in the disease is a primary association.

The contradictory, double role of telomeres in cancer is very interesting. On the one hand telomere dysfunction promotes tumour formation, but on the other hand, functional telomeres are required for tumour progression. In most human somatic cells telomerase is not active, meaning cells have a limited replicative capacity. As cells divide, telomeres shorten with each cell division until they reach a critical length and enter a senescent state (Harley et al., 1990). Tumour cells, on the other hand have an unlimited replicative capacity. They bypass the signal to stop dividing. In order to take on the 'immortal' nature, cells must find a way to maintain chromosome ends. In approximately 90% of cancers, telomerase activation is the method by which

cells achieve this (Kim et al., 1994). In the remaining cancers, alternative mechanisms are utilised, the most well described being the ALT method of telomere amplification by recombination. However, cancer is a complex disease with multiple stages in its progression. Telomerase reactivation occurs late in tumorigenesis (Blasco and Hahn, 2003). Despite the eventual requirement of being able to maintain chromosome ends to allow the proliferative nature of tumour cells, the actual loss of telomere DNA plays an important role at the earlier stages of malignant transformation (Maser and DePinho, 2002). Genetic instability is a hallmark of cancer. Telomere shortening results in deprotected chromosome ends, leading to genomic instability following cycles of chromosome fusion and breakage. While most cells die within this period termed 'crisis', populations of cells may survive the genetic alterations with the ability to take on the many features of cancer and gain the ability to stabilise chromosome ends.

Another disease with close associations with telomere biology is the rare multisystem disorder, dyskeratosis congenita (DKC). Forms of DKC are associated with mutations in the telomerase accessory factor, Dyskerin (DKC1) and the telomerase RNA template (hTERC) (Heiss et al., 1998; Knight et al., 1999; Mitchell et al., 1999; Vulliamy et al., 2001).

Suggestions have been made that aging could, in part, be brought about by telomere attrition. Certainly, in cultured cells, telomere shortening correlates with proliferative failure, which can be overcome by activation of telomerase and subsequent telomere lengthening (Bodnar et al., 1998). It is conceivable that cellular aging is directly correlated to aging of an organism; the inability of a tissue to regenerate through cell division would lead to organ failure, a mark of aging.

1.5 Fission yeast as a model organism

Fission yeast has proved to be a useful organism for studying chromosome biology. The ease of growth and genetic manipulation make it very attractive for use in research. In evolutionary terms, fission yeast is as distant from humans as from budding yeast, making it an interesting comparison for looking at conserved pathways and structures. The genome organisation is

similar to that of humans with large centromeres formed from a complex, heterochromatic structure, unlike the budding yeast counterpart. Fission yeast has also proved a very useful model for telomere biology. The small number of chromosomes means there are only six telomeres. The major telomere binding protein, Taz1, is the only known ortholog of the human myb-domain telomere binding proteins, TRF1 and TRF2. Unlike in other organisms, however, disruption of Taz1 is not lethal. Finally, the small number of chromosomes means we are able to study a unique situation; strains maintaining chromosomes in the absence of telomeres.

1.6 Thesis aims

Upon embarking on my PhD work, I set out to gain a better understanding of the role telomeres play in the survival of DNA damage. To address the topic I utilised fission yeast strains that lack telomeres, having survived by circularising each of the three chromosomes. In carrying out the investigations I uncovered two strains surviving by novel mechanisms following loss of terminal telomere DNA sequences upon disruption of telomerase. My work then turned to characterising these strains to gain a better understanding of the survival mechanism. In doing so, I took a step towards unravelling the role of telomeres and chromosome topology in surviving DNA damage.

2 Materials and methods

2.1 Yeast Strains and media

Fission yeast strains used in this study are listed in Table 1. Many strains were created by mating, selecting on appropriate media, or the one-step gene replacement method using a kanMX6 cassette (Bahler et al., 1998). *trt1* knockout strains were created using a *trt1::his3* knockout fragment created by PCR amplification from genomic DNA of a *trt1*Δ strain kindly provided by T. Nakamura. The internal telomere strain was created by replacing *ura4* with the *LEU2*-telo fragment amplified by PCR from the plasmid pIRT2-telo, selecting for loss of *ura4* on 5'-FOA.

Media and growth conditions were as described previously (Moreno et al., 1991). Cultures were grown at 32°C in rich media (YE4S) unless otherwise indicated. Plasmid containing strains were grown under conditions selecting for the appropriate marker. *rad3*^{ts} strains were grown at 25°C for the permissive temperature and 36°C for the restrictive temperature.

For nitrogen starvation experiments, cultures were grown to log phase in EMM, washed twice in EMM without NH4Cl, and resuspended at a density of 1x106 cells/ml in EMM without NH_4Cl. Cells were starved for 24-72 hours and a sample ethanol fixed for FACS analysis.

Table 1 *Schizzosacharomyces pombe* strains used in this study

JCF number	genotype	mating type	Lab
1	wild type	h^-	Cooper lab
2	wild type	h^+	Cooper lab
28	*taz1::ura4 ura4-D18*	h^-	Cooper lab
108	*ade6-M210 his3-D1 leu1-32 ura4-D18*	h^-	McIntosh lab
170	*rik1::ura4 ade6-M210 leu1-32 ura4-D18*	h^+	Cooper lab
214	*bub1::ura4 ade6-M8 leu1-32 ura4-D18*	h^+	Javerzat Lab
221	*rad3::ura4 ade6-M210 leu1-32 ura4-D18*	h^+	Ishikawa Lab
205	*taz1-GFP-kan*	h^+	Cooper lab
329	*clr4::kan ade6-469*	h^-	Cooper lab
443	linear *trt1::his3 taz1::ura4 ura4-D18 leu1-32 his3-D1 ade6-M210*	h^-	Cech Lab
444	circular *trt1::his3 taz1::ura4 ura4-D18 leu1-32 his3-D1 ade6-M210*	h^-	Cech Lab
442	circular *trt1::his3 leu1-32 his3-D1 ura4-D18 ade6-M210*	h^-	Cech Lab
905	*ura4::telo-LEU2 his3-D1 leu1-32*	h^+	This Study
908	*ura4::telo-LEU2 taz1-GFP-kan his3-D1*	h^+	This Study
909 'C1'	*ura4::telo-LEU2 trt1::his3 his3-D1 leu1-32*	h+	This Study
909 'X1'	*ura4::telo-LEU2 trt1::his3 his3-D1 leu1-32*	h+	This Study
909 'X2'	*ura4::telo-LEU2 trt1::his3 his3-D1 leu1-32*	h+	This Study

910	*ura4::telo-LEU2 trt1::his3 taz1-GFP-kan his3-D1*	h^+	This Study
911	*trt1::his3 taz1-GFP-kan his3-D1*	h^+	This Study
912	*trt1::his3 ade6-M216 his3-D1 leu1-32 ura4-D18*	h^+	This Study
914	*trt1::his3 rik1::ura4 leu1-32 his3-D1 ura4-D18 ade6-M210*	h^-	This Study
918	*rad3::ura4 tel1::LEU2 ade6-M210 leu1-32 ura4-D18*	h^{90}	Ishikawa Lab
932	*rad3^{ts} leu1-32 ade6-M? his3-D1*	h^-	Toda Lab
934 'H1'	*trt1$^-$ ade6-M210*	h^-	Cech Lab
945	'X1' *taz1-GFP-kan*	h^+	This Study
946	'X2' *taz1-GFP-kan*	h^+	This Study
947	*pot1::ura4 ade6$^-$ leu1-32 ura4-D18*	h^+	Cooper Lab
948	'X1' *taz1::ura4*	h^+	This Study
949	'X2' *taz1::ura4*	h^+	This Study
950	*his4::I-Sce1-kan*	h^-	This Study
951	*his4::I-Sce1-kan* circular *trt1::his3 leu1-32 his3-D1 ura4-D18 ade6-M210*	h^-	This Study
952	*his4::I-Sce1-kan* 'X1'	h^+	This Study
953	*his4::I-Sce1-kan* 'X2'	h^+	This Study

2.2 Yeast transformations

50ml cultures were grown to log phase in YE4S media. Cells were pelleted, washed in 50ml ddH_2O, followed by 1ml ddH_2O, then 1ml LiOAc solution (0.1M LiOAc, 10mM Tris-HCl (pH8), 1mM EDTA). Cells were resuspended in 200µl LiOAc solution and 100µl used for each transformation. Salmon sperm DNA (10µg/ml, Stratagene) was boiled for 5 mins then placed on ice. 2µl was added to cell mix along with transforming DNA (~5mg PCR product or ~1mg supercoiled plasmid DNA). Cells were incubated with the DNA at room temperature for 10 mins. 260µl PEG4000 (40% in LiOAc solution) was added, mixed gently by pipetting, then incubated for 30-60 mins at 30°C (25°C for temperature sensitive strains). 43µl pre-warmed DMSO was added and the cells heat-shocked at 42°C for 5 mins. Cells were pelleted, washed in 1ml ddH_2O then resuspended in 500µl ddH_2O. 250µl (200µl for plasmid transformations) was plated on the appropriate media and incubated at 32°C (25°C for temperature sensitive strains) for selection. For Kan^r selection, cells were first plated onto YE4S and incubated overnight before replica plating onto YE4S + G418. Transformants were picked, restreaked onto selective media and their genotype verified by PCR and/or southern analysis.

2.3 Cytological analysis

Cell morphology was analysed by growing cells to log phase in rich media at 32°C. Cells were visualised using light microscopy or differential interference contrast microscopy (DIC). Nuclear morphology was visualised by fixing cells in 70% ethanol, rehydrated in ddH_2O and stained with 4', 6-diamidino-2-phenylindole (DAPI, Vectashield, Vector Laboratories). DAPI staining was visualised on a Nikon Eclipse E600 flourescence microscope. All images were taken on a MTI 300T-RC CCD camera using Scion Images software.

2.4 Viability and sensitivity assays

2.4.1 Chronic treatment on plates

Cells were grown in liquid culture to log phase, their density estimated using a haemocytometer. For dilution assays, cultures were adjusted to 1×10^7

cells/ml as the starting concentration and serially diluted by a factor of 5 in a 96 well microtiter plate. 5μl of each dilution was spotted onto the appropriate media containing the indicated concentration of drug and grown under the appropriate conditions.

For viability curves, cultures were diluted and 300 or 500 cells were spread onto the appropriate media containing the indicated concentration of drug and grown under the appropriate conditions. Colonies were counted after 4 days growth and viability displayed as the ratio of colonies counted under experimental conditions compared with colonies counted under optimal conditions.

2.4.2 Acute treatment in liquid culture

For acute treatment, cells were grown to log phase in the absence of the drug. Cultures were split into the appropriate sample size and incubated with the drug in a shaking incubator at 32°C (MMS and bleomycin). Cultures were either incubated in increasing concentrations of drug for 3 hours or in one concentration and samples removed at 20 minute time points. Cells were washed twice in ddH_2O and resuspended in ddH_2O. For γ-radiation, cells were treated in liquid with the appropriate dose. Cell density was estimated with a haemocytometer, 300 or 500 cells plated in triplicate on rich media and incubated at 32°C. Colonies were counted after 4 days growth and viability displayed as the ratio of colonies counted under experimental conditions compared with colonies counted under optimal conditions.

For acute treatment with UV radiation, cells were serially diluted as described above (Chapter 2.4.1), plated onto rich media and treated with UV radiation of the plates. Immediately following treatment, plates were wrapped in aluminium foil to prevent any possible photoreactive repair process (thought not to be an issue in *S. pombe*, but not confirmed). Plates were incubated at 32°C for 4 days.

2.5 Pulsed field gel electrophoresis

Cells were grown in 25ml cultures to log phase. Cells were pelleted, washed in 1ml SP1 buffer (50mM citrate/phosphate (pH 5.6), 40mM EDTA, 1.2M sorbitol) and resuspended in 1ml SP1 buffer. Cell density was estimated using a haemocytometer. Meanwhile, cells were treated with 0.6mg/ml zymolyase-100T (ImmunO) in SP1 buffer for 10-30 mins at 37°C to spheroplast. Spheroplasts were checked by looking for the appearance of 'ghost' cells upon treatment of 10μl of cells with 1% SDS on a microscope slide. Zymolyase was removed by spinning down at 3000rpm for 1 min. Sheroplasts were gently resuspended in 1% low melting point agarose in TSE (10mM Tris-HCl (pH 7.5), 0.9M sorbitol, 45mM EDTA) to give a final concentration of 1 x 10^9 cells/ml and immediately dispensed into 100μl plug moulds. Plugs were allowed to solidify at 4°C for 10 mins. Plugs were transferred to tubes containing 3ml 0.25M EDTA, 50mM Tris-HCl (pH 7.5), 1% SDS and incubated at 55°C for 90 mins. The solution was removed and replaced with 0.5M EDTA (pH 9.5), 1% lauryl sarcosine, 1mg/ml proteinase K and incubated at 55°C for 48 hours, changing the solution after 24 hours. Plugs were washed twice in a large volume of T10xE (10mM EDTA, 10mM Tris-HCl (pH 7.5)) for 30 mins at 25°C, followed by 1 hour in T10xE containing 0.04mg/ml PMSF at 50°C, then twice more for 30 mins in T10xE at 25°C. Plugs were stored at 4°C in 0.5M EDTA, 10mM Tris-HCl (pH 9.5) until used.

2.5.1 Whole chromosome analysis

Plugs were pre-equilibrated in 1 x TAE on ice for 1 hour and loaded into a 0.8% agarose gel in 1 x TAE. PFGE was performed on a BioRad CHEF DR-III system in 1 x TAE at 14°C using the following program:

Step 1, 24 hours at 2v/cm, 96° angle, 1200 second switch time
Step 2, 24 hours at 2v/cm, 100° angle, 1500 second switch time
Step 3, 24 hours at 2v/cm, 106° angle, 1800 second switch time.

The gel was stained in ethidium bromide for 30 mins, followed by destaining in ddH_2O for 1 hour to overnight.

2.5.2 NotI restriction fragment analysis

Plugs were washed twice in T10xE for half an hour at 25°C if digested from storage buffer. Plugs were incubated at 37°C in 2x NE Buffer 3 (NEB, 10x buffer: 0.5M Tris-HCl, 1M NaCl, 100mM $MgCl_2$, 10mM Dithiothreitol, pH 7.9 @ 25°C), supplemented with 20μg/ml BSA for 1 hour followed by 1x NE Buffer 3 with 10μg/ml BSA for 1 hour. 100 units NotI enzyme were added and plugs incubated at 37°C overnight. Plugs were equilibrated in 0.5x TBE on ice for 1 hour and loaded into a 1% agarose gel in 0.5x TBE. PFGE was performed on a BioRad CHEF DR-III system in 0.5x TBE at 14°C using the following program:

24 hours at 6v/cm, 120° angle, 60-120 second switch time.

The gel was stained in ethidium bromide for 30 mins, followed by destaining for 1 hour to overnight in ddH_2O.

2.5.3 SfiI restriction fragment analysis

Plugs were washed twice in T10xE for half an hour at 25°C if digested from storage buffer. Plugs were incubated on ice in 2x NE Buffer 2 (NEB, 10x buffer: 0.1M Tris-HCl, 0.5M NaCl, 100mM $MgCl_2$, 10mM Dithiothreitol, pH 7.9 @ 25°C), supplemented with 20μg/ml BSA for 1 hour followed by 1x NE Buffer 2 with 10μg/ml BSA for 1 hour. 100 units SfiI enzyme were added and plugs incubated at 50°C for 6 hours. Plugs were equilibrated in 0.5x TBE on ice for 1 hour and loaded into a 1% agarose gel in 0.5x TBE. PFGE was performed on a BioRad CHEF DR-III system in 0.5x TBE at 14°C using the following program:

24 hours at 6v/cm, 120° angle, 60-120 second switch time.

Gels were stained in ethidium bromide for 30 mins, followed by destaining for 1 hour to overnight in ddH_2O.

2.5.4 I-SceI digestion

Plugs were washed twice in T10xE for half an hour at 25°C if digested from storage buffer. Plugs were incubated at 4°C in 0.1M diethanolamine (pH 9.5) overnight. One or half a plug was incubated on ice in 160µl 0.1M diethanolamine (pH 9.5), 0.001M DTT, 0.02mg/ml BSA for 1 hour. 2µl 'enhancer' was added along with 30-40 units I-SceI (both Roche) and allowed to diffuse into the plugs on ice for 2 hours. $MgCl_2$ was added to a final concentration of 8mM to activate the enzyme and incubated at 37°C for 1 hour. Plugs were pre-equilibrated in 1 x TAE on ice for 1 hour and loaded into a 0.8% agarose gel in 1 x TAE. PFGE was performed on a BioRad CHEF DR-III system in 1 x TAE at 14°C using the following program:

Step 1, 24 hours at 2v/cm, 96° angle, 1200 second switch time
Step 2, 24 hours at 2v/cm, 100° angle, 1500 second switch time
Step 3, 24 hours at 2v/cm, 106° angle, 1800 second switch time.

The gel was stained in ethidium bromide for 30 mins, followed by destaining in ddH_2O for 1 hour to overnight.

2.6 Genomic DNA preparations

(From (Burke et al., 2000)). 10ml cultures in YE4S (or selectable media for strains containing plasmids) were grown overnight to saturation. Cells were collected by centrifugation and transferred to an eppendorf tube in 500µl ddH_2O. Cells were collected by centrifugation for 5 seconds. Cells were resuspended in 200µl 'smash prep' buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-HCl (pH 8), 1mM Na_2EDTA). 200µl phenol:chloroform:isoamyl alcohol was added along with 0.3g of acid washed beads and vortexed for 3-4 mins. 200µl TE (pH 8) was added and tubes centrifuged for 5 mins. The aqueous layer was transferred to a fresh tube, 1ml 96% ethanol added and mixed by inversion. Tubes were centrifuged for 2 mins and the supernatant discarded. The pellet was resuspended in 400µl TE plus 3µl of a 10mg/ml solution of RNase A and incubated for 5 mins at 37°C. 10µl 4M ammonium acetate was added plus 1ml 96% ethanol and mixed by inversion. Tubes were centrifuged for 2 mins, supernatant discarded and

pellet air dried at room temperature. The pellet was resuspended in 50μl TE. 10μl of each sample was used for southern analysis or 1μl for PCR.

2.7 Southern analysis

DNA was digested with the appropriate restriction enzyme and run in 1 x TAE on a 1% agarose gel containing 0.03mg/ml ethidium bromide. These gels or pulsed field gels were then treated for Southern transfer. Gels were incubated in 0.25N HCl for 15 mins, followed by 30 mins in Blot 1 solution (20g NaOH, 87.6g NaCl in 1l H_2O), then 60 mins in Blot 2 solution (77g NH_4Ac, 0.8g NaOH in 1l H_2O) and finally washed for 5 mins in 6 x SSC. During this time, the membrane was prepared. Duralon-UV membrane (Stratagene) was cut to size and soaked in ddH_2O for 10 mins, then Blot 2 solution for 10 mins. To set up the dry transfer, a stack of paper towels, about 3 inches thick, were placed on the bench. On top of this, three pieces of 3mm Whatmann paper were placed, followed by the treated membrane and gel, wells up. The stack was covered with cling film followed by a glass plate to ensure even distribution of weight. The stack was weighed down with 2-3 full 500ml Duran bottles. Transfer was allowed overnight. The stack was dismantled and DNA was crosslinked to the membrane with a Stratalinker (Stratagene) using 1200 microjoules (x100). The membrane was then pre-hybridised in Church Gilbert buffer (1% BSA, 1mM EDTA, 7%SDS, 0.5M $NaHPO_4$ (pH 7.2) (to make 1 litre at 1M, 134g Na_2HPO_4-$7H_2O$ and 4ml 85% H_3PO_4)) for 2 hours at the appropriate temperature. For oligo probes, this was at 45-65°C, and for random primed probes, 65°C.

2.7.1 Oligo probe preparation

For telomere oligo probes, 2μl (50μM stock) of each oligo (ABT and BCT (sequence listed in Table 2)) was incubated with 1μl 10 x kinase buffer, 1μl T4 polynucleotide kinase, 1μl H_2O and 3μl γ-^{32}P dATP at 37°C for 1 hour. 90μl TE was added and placed over a pre-equilibrated Sepharose G-25 spin column (Amersham). Probe was heated at 100°C for 5 mins to denature before adding 50μl to hybridisation mix. Hybridisation was allowed overnight at the appropriate temperature.

2.7.2 Random primed probe preparation

PCR or plasmid restriction fragments were gel purified and prepared using a random prime labelling kit (Stratagene). 25ng DNA in 24μl ddH_2O was mixed with 10μl random oligonucleotide primers and heated at 100°C for 5 mins. 10μl 5 x dCTP buffer was added with 5μl α-^{32}P dCTP and 1μl Exo(-) klenow polymerase (5u/μl). Tubes were incubated at 37°C for 10 mins. 2μl stop mix and 48μl TE was added and placed over a pre-equilibrated Sepharose G-25 spin column. Probe was heated at 100°C for 5 mins to denature before adding 50μl to hybridisation mix. Hybridisation was allowed overnight at 65°C.

Following hybridisation, probe was poured off and membrane rinsed twice in 100ml wash solution (2 x SSC, 0.1% SDS), followed by incubation in 100ml wash solution for 30 mins at hybridisation temperature. Membrane was wrapped in cling film and put down on a phosphorimager screen for 4-72 hours. Signal was detected on a Molecular Dynamics Storm 860 phosphorimager system.

2.7.3 Removal of probes for re-use of membranes

Membrane was incubated in 0.4M NaOH at 42°C for 30 min, followed by 30 mins in 0.1x SSC, 0.1% SDS, 0.2M Tris (pH 7.5) at 42°C. Membrane was then preincubated in Church Giltert solution before re-probing (as above).

2.8 BAL-31 digestion

30μl genomic DNA was incubated with linear plasmid DNA and 20 units BAL-31 in 1x BAL-31 buffer (NEB; 20mM Tris-HCl, 600mM NaCl, 12mM $CaCl_2$, 12mM $MgCl_2$,1mM EDTA pH 8 @ 25°C) in a total reaction volume of 130μl at 30°C for the indicated time. Reactions were heat inactivated at 65°C in the presence of EGTA for 10 mins. DNA was phenol chloroform extracted, ethanol precipitated and pellets allowed to air dry before resuspending in ddH_2O. Half the DNA was run on a 1% agarose gel to assess BAL-31 digestion of DNA by a decrease in size and intensity of the linear plasmid. The remainder of the DNA was digested with the appropriate restriction

enzyme. Samples were run out on a 1% agarose gel in 1xTAE and transferred to a membrane for Southern analysis.

2.9 RNA preparations

(From (Schmitt et al., 1990)). All procedures carried out in 'RNase free' conditions. 10ml overnight cultures were grown in YE4S. Cells were pelleted and resuspended in 400µl AE buffer (50mM NaOAc (pH 5.3), 10mM EDTA). Cells were transferred to a microcentrifuge tube and 40µl 10% SDS added. Tubes were vortexed and an equal volume of phenol added. Tubes were vortexed and incubated for 4 mins at 65°C. The mixture was then rapidly chilled in a dry ice/ethanol bath until phenol crystals appeared, then centrifuged for 2 mins. The upper, aqueous layer was transferred to a fresh tube and extracted with phenol/chloroform at room temperature for 5 mins. 40µl NaOAc (pH 5.3) was added with 2.5 volumes of 96% ethanol to precipitate the RNA. The RNA was pelleted, washed with 80% ethanol and air dried before resuspending in 20µl RNase free water.

2.10 Northern analysis

10µg RNA was run on a formaldehyde gel (1% agarose, 1xMOPS, 6% Formaldehyde. 10xMOPS: 20.9g/L MOPS, 50mM Sodium acetate, 10mM EDTA, adjusted to pH 7 with NaOH). Samples were mixed with sample buffer (2µl RNA sample, 5µl Formamid, 2µl 37% Formaldehyde, 1µl 10xMOPS, 0.05µl Ethidium Bromide), heated for 5 mins at 95°, and chilled on ice before being loaded onto the gel. Gel was run in 1x MOPS buffer at 120 volts until bromophenol blue reached the bottom of the gel. RNA was visualised on a UV transilluminator as a loading control. Prior to transfer, gel was washed in RNase free water. Northern transfer was set up in 1x MOPS on a stack of paper towels onto a Duralon UV membrane. Transfer was allowed overnight. RNA was UV crosslinked onto the membrane with a Stratalinker (Stratagene) using 1200 microjoules (x100). The membrane was then prehybridised in 1M NaCl, 10% Dextran Sulphate, 1% SDS at 60°C. The probe was prepared as with Southern analysis and added to the hybridisation mix. Hybridisation occurred overnight at 60°C. Membrane was washed twice for 30 mins in 2x SSC, 1% SDS at 60°C. Membrane was wrapped in cling film and put down

on a phosphorimager screen for 4-72 hours. Signal was detected on a Molecular Dynamics Storm 860 phosphorimager system.

2.11 FACS analysis

1ml samples from cultures were fixed with 70% ethanol and stored at 4°C until analysed. 300µl cells were mixed with 3ml 50mM Na citrate in a 5ml falcon tube to rehydrate. Cells were collected by centrifugation at 2000rpm for 5 mins. The pellet was resuspended in 500µl 50mM Na citrate containing 0.1mg/ml RNase A and incubated at 37°C for 2 hours. 500µl 50mM Na citrate containing 4µg/ml propidium iodide was added and cells processed immediately or stored overnight at 4°C in the dark for processing the following day. Before processing, cells were sonicated for 45 sec. DNA content was analysed on a Becton Dickinson FACScan.

2.12 PCR

PCR was performed using standard reagents and conditions. High fidelity long template Expand Taq Polymerase (Roche) was used to amplify fragments for cloning, knockouts and tagging. 'Generic' Taq polymerase supplied by Cancer Research UK stores was used for other reactions. PCR primers are listed in Table 2.

2.12.1 Reaction conditions for standard PCR

1µl template DNA
5µl Expand Long Template Buffer 1 (Roche)
2µl $MgCl_2$ (2mM)
3.3µl dNTP mix (3mM)
1µl each primer (10µM)
0.75µl Taq polymerase
35.95µl ddH_2O

2.12.1.1 PCR Program

Step 1	95°C	5 mins
Step 2	95°C	30 secs
Step 3	45-55°C	1 min (depending on Tm of primers)

Step 4	68°C	1 min/kb extension
Repeat steps 2-4 x30		
Step 5	68°C	5 mins
Step 6	4°C	∞

2.12.2 Reaction conditions for use with long primers

1µl template DNA (14ng for Bahler method plasmids)

5µl Expand Long Template Buffer 1 (Roche)

5µl $MgCl_2$ (25mM)

2.5µl dNTP mix (10mM)

1µl each primer (4µM)

1µl Expand Long Template Taq polymerase

33.5µl ddH_2O

2.12.2.1 PCR program

Step 1	95°C	5 mins
Step 2	95°C	1 min
Step 3	50°C	2 mins
Step 4	72°C	2 mins
Repeat steps 2-4 x5		
Step 5	95°C	1 min
Step 6	56°C	2 mins
Step 7	72°C	2 mins
Repeat steps 5-7 x35		
Step 8	72°C	5 mins
Step 9	4°C	∞

Table 2 PCR primers and Southern probe oligos used in this study

Primer name	Sequence (5'-3')
trt1-u	ATGTGGGCAGTAGTCAGCAA
trt1-d	CTACAACTCCTTTAACGCGG
Ver trt1 ko-1	ACTCCCGTTTAATGGGCATG
Ver trt1 ko-2	CTTTCTAGAAGTAGCCCTCA
Ver trt1 ko-3	TCCTTAGTGGTGGTAATCCG
Ver trt1 ko-4	TATGGCGAAGGGAACAGTTG
TelInt ura4-u	GCTAGAGCTGAGGGGATGAAAAATCCCATTGCCAAGGAATTGTTGGCTTTGATGGAAGAAAAGCAAAGCA
	ACTTGTCAGTCGCGGTCGATTTGACGAAGATTTCAGAGGTCGCCTGACGC
TelInt ura4-d	GCTGAGAAAGTCTTTGCTGATATGCCTTCCAACCAGCTTCTCTATATCTCTTGGCTTCGACAACAGGATTA
	CGACCAGCTCCATAGACTCCACGACCAACTTGGAATTCCGAGCTCGGTA
ver tel ura4-1	GCCTTCTGACATAAAACGCC
ver tel ura4-2	TCTTACCGTATTGTCCTACC
ver tel int-1	CGACGTAGTCGACAAGCTTT
ver tel int-2	CTTAATGGCTTCGGCTGTGA
act1-u	TTATTGATAATGGCTCTGGT
act1-d	ATGGGAACAGTGTGGGTAAC
telo helic. dh-u	ACTGTGTCTACGATGTATGG

telo helic. dh-d	CTGAGCGACATGTCTTCCAA
taz1-u	CACCATACAATCGAGGGCAG
taz1-d	GCTTCACTCATTTACGATTC
taz1-int	GCAGTAAGCTGATTGCGAAG
kan1-u	ATGCATCATCAGGAGTACGG
kan1-d	ACGGTTTGGTTGATGCGAGT
ura4-u	CCCTCAGCTCTAGCTGAATA
ura4-d	TAGAGAAGCTGGTTGGAAGG
His4-I-Sce-I-u	GGCTGTATGCGTTGGAATCCAAGCATTATTCGAGGGGTCTGTTGAAGCACCACATTCTAAAGGGTTGGGC
	GTATTTCCGGGGTTAGTCCAAAGGTTTGACCGGATCCCCGGGTTAATTAA
His4-I-Sce-I-d	CACGAGTAAGTTCAACAACATCAATATCACGATATTCACGACCTCCTTTAACGGTGCACTGATACCAACAA
	TATGCTTCTCCTAGGGATAACAGGGTAATGAATTCGAGCTCGTTTAAAC
Ver his4-I-Sce1 cut-u	TCGATCCTTAATCAATGCCG
Ver his4-I-Sce1 cut-d	AGACAAAGCATAGCGACGCA
pIRT2-seq1	CCTGACCAACGTGGTCACCT
pIRT2-seq2	TACCACCGAAGTCGGTGATG
pIRT2/telo seq1	GACCTGACCATTTGATGGAG
pIRT2/telo seq2	CCCTTGTAAATCATCTGATG
L-u	GCCACATAAAAAGTGCGATTGGCGG
L-d	GTGGGAGGCTATAACGGTCAGTTCC

M-u	CGACACTGACATTGTCACACTTTCC
M-d	CGACTTCATGTTTACTTCCAAGCC
I-u	CCGATTGAGAAGTATTGTAATAGG
I-d	CAATCATCTTTCCATGAACCGGC
C-u	CCTAGTCAATCCACCTACAGAGG
C-d	CCATAAAAGTAGCAGCCAGATCCCC
his4-u	GACGTCTTCTATGCGACAAG
his4-d	AGCTACCCAATTTCATCCGG
cdc3-u	CCTAGTATGTTTGGCACTGG
cdc3-d	CAAATCAAGCAACAGCTCCC
ABT	GATCGGGTTACAAGGTAACGTGGTTACACGGTTACAGATC
BCT	GATCTGGTTACACGGTTACAGGTTACAGGTTACAGGGATC

3 Strains with circular chromosomes show growth defects and hypersensitivity to DNA damaging agents

3.1 Introduction

The generalisation that eukaryotic chromosomes have evolved from the circular genomes of prokaryotes into organisms with linear chromosomes poses a potential problem; the evolution of natural chromosome ends. Ends that need to be protected from degradation, prevented from being detected as broken DNA, and synthesised using different machinery to the conventional replication machinery of a cell. As a consequence, organisms with linear chromosomes have developed a strategy to deal with the presence of chromosome ends; telomeres. If the presence of ends poses such a potential problem to a cell or an organism, why have ends at all? As explored in greater detail in Chapter 1, telomeres play a diverse role in cell biology and maintenance of genome integrity. Their known function outside of the basic role of 'being the ends of the linear genomes' is becoming more diverse. But are these roles of telomeres important because of the linearity of the genome, or do telomeres themselves have roles that are important, conferring advantages to a linear genome? Why do we have linear chromosomes?

The linearity of chromosomes is problematic to the conventional DNA replication machinery. Removal of the terminal RNA primer required for lagging strand synthesis following replication leads to incomplete synthesis of the 5' end. Similarly, lack of template on the leading strand prevents synthesis of the 3' overhang, an inherent part of the telomere structure. This issue is overcome by the presence of telomerase, adding terminal sequences to the chromosomes and synthesising the overhang. Circular DNA molecules, however, do not have this problem. Replication can be continued round the whole of the molecule, and there is no overhang that should be replicated.

Another important role of telomeres is to differentiate natural chromosome ends from those of DNA double strand breaks. Once again, it is only through

the presence of chromosome ends that this problem arises. Maintaining the genetic information within a circular genome should bypass the requirement for these so-called 'anti-checkpoint' and 'anti-repair' functions of telomeres.

As discussed in greater detail in Chapter 1, a role of telomeres in meiosis is also being explored, but the details remain largely speculative. During meiosis telomeres cluster into the so called 'bouquet structure' (Chikashige et al., 1994). The role for such a structure is largely unknown, but suggestions that it promotes homologous pairing, leading to recombination are attractive. Fission yeast strains lacking telomeres, surviving with circular chromosomes, are defective in meiosis (Naito et al., 1998; Nakamura et al., 1998). Whether this defect is due to a lack of telomere sequence, the topology of the chromosomes, or a combination of the two is unknown. A strong possibility is that meiotic recombination of circular chromosomes results in dicentric circular chromosomes, however, such a dicentric could, in principle, be resolved.

Previous work from our lab implicates the telomere binding protein, Taz1, with a role in survival following DNA double strand breaks (Miller and Cooper, 2003). Evidence in other organisms may also suggest a role of telomeres or associated proteins in repair of DNA damage. Mice lacking telomerase display a general sensitivity to alkylating agents and γ-irradiation, but only in late generations when telomeres are shortened (Gonzalez-Suarez et al., 2003; Goytisolo et al., 2000; Wong et al., 2000). Telomerase negative human cell lines are sensitive to ionising radiation due to dysfunctional telomere structure, rather than length (Rubio et al., 2002).

In this chapter we explore some of the problems in fission yeast that arise from having chromosomes without telomeres, surviving through circularisation of each of the three chromosomes. We characterise and document more fully some of the previously described problems associated with these so-called 'circular strains'. We also describe a further defect associated with these strains; a general sensitivity to damaging agents. The common defect of damage sensitivity seen in strains lacking telomeres and strains with defective

telomeres through disruption of Taz1, lead us to question and investigate the role of telomeres in survival following DNA damage.

3.2 Circular strains are viable but sick

Despite the previous description of *trt1*Δ strains surviving following loss of all telomeric DNA and most of the subtelomeric elements (Nakamura et al., 1998), little characterisation of these strains has been reported. While circular strains are viable, they grow slowly and exhibit DAPI staining patterns suggestive of chromosome segregation defects (Figure 3.1). The elongation phenotype observed is indicative of checkpoint activation in response to some kind of damage; DNA breaks, replication defects or spindle assembly problems. Within an exponentially growing culture of 'circular' cells, many dead cells are observed, primarily dying as non-elongated and often partially divided cells (a cut phenotype) (Figure 3.1).

A similar phenotype is also observed in other strains having survived by chromosome circularisation following loss of the telomere and telomere associated DNA. Fission yeast strains lacking the telomere end binding protein, Pot1, lose telomere and subtelomeric elements rapidly, surviving through chromosome circularisation (Baumann and Cech, 2001). Despite previous reports that *pot1*Δ survivors display a wild type morphology, we observe the morphology to be reminiscent of circular *trt1*Δ cells; an elongated phenotype, many dead cells and chromosome segregation defects (Figure 3.1 C). Circular survivors also display multiple septa, possibly suggesting a problem with the septation initiation network (SIN) (Figure 3.1). Following mitosis, if SIN does not turn off effectively, cells become elongated and multinucleated, and go through cycles of ring formation and septation without cell cleavage, resulting in mutiseptated cells. However, the circular strains are not reminiscent of *bona fide* SIN mutants; while we see mutiseptated cells, we do not observe multinucleated cells. Further analysis of these strains should be carried out to understand the basis behind the multiseptated phenotype observed in circular strains.

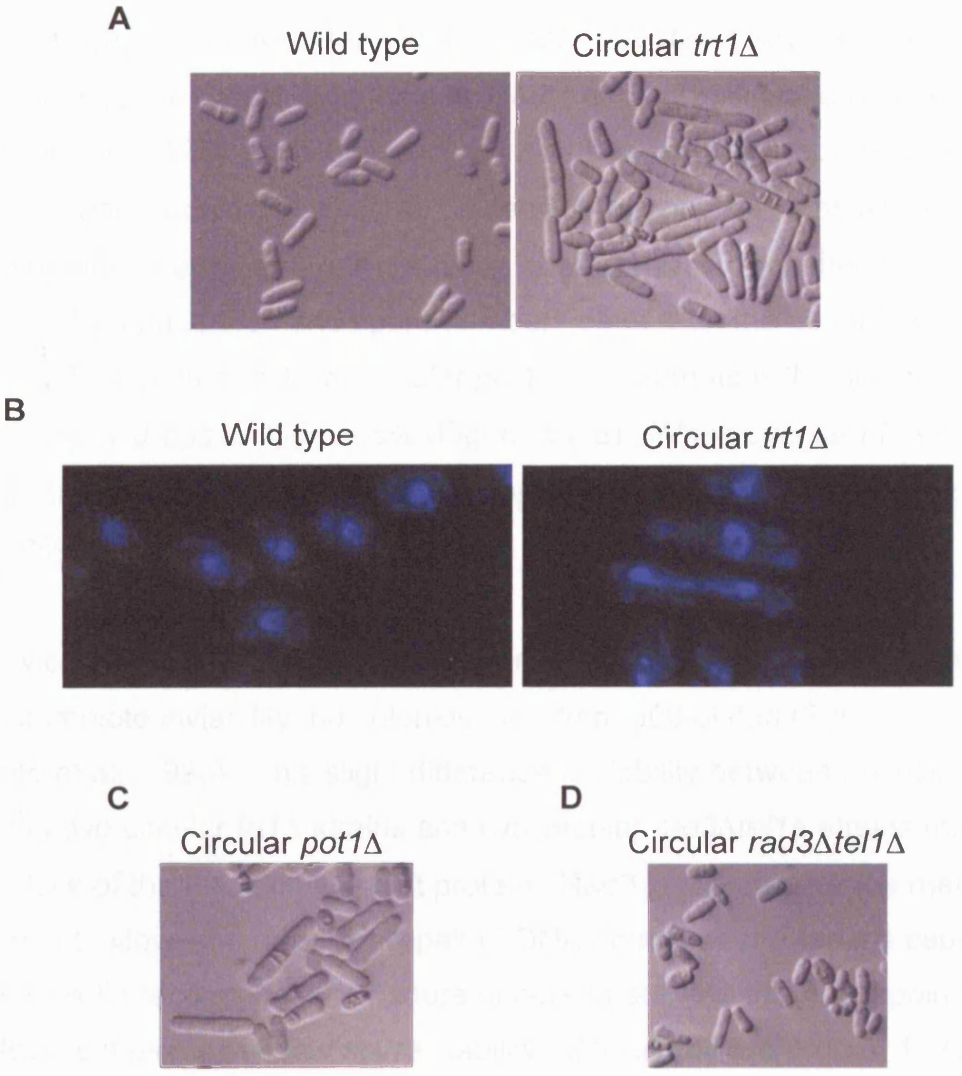


Figure 3.1 Strains with circular chromosomes are viable but sick

(A) DIC pictures demonstrate *trt1*Δ strains harbouring circular chromosomes are elongated. There are also many dead cells and cells with multiple septa.
(B) DAPI staining shows circular *trt1*Δ strains exhibit chromosome segregation defects.
(C) Circular *pot1*Δ strains also have elongated cells and cells with multiple septa.
(D) Circular *rad3*Δ*tel1*Δ strains are not elongated due to loss of checkpoint function, but there are cells with multiple septa and many dead cells in a culture.

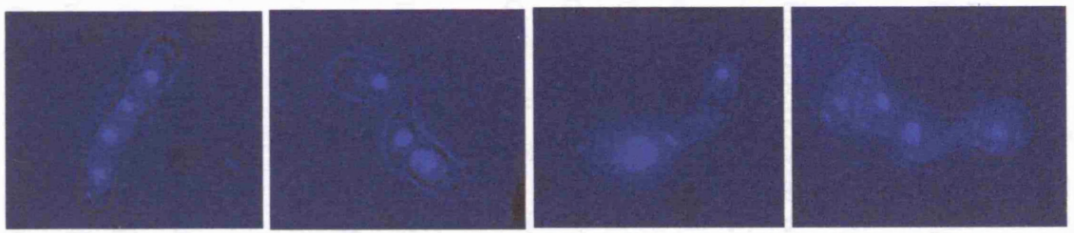
3.3 Circular strains are defective in meiosis

It has previously been reported that *trt1*Δ and *rad3*Δ*tel1*Δ strains with circular chromosomes are defective in meiosis (Naito et al., 1998; Nakamura et al., 1998). Meiotic ability of circular *pot1*Δ strains has not been reported, but a similar situation would be expected. Indeed, when *pot1*Δ strains are mated with either linear or other circular strains, aberrant asci form, often with abnormal looking spores and spore numbers other than the usual four (Figure 3.2 A). When circular *trt1*Δ or circular *pot1*Δ strains mate with a linear strain, spore viability drops to 15% - 18% (Figure 3.2 B). When circular *trt1*Δ mates with another circular *trt1*Δ strain of the opposite mating type, viability drops to below 6% (Figure 3.2 B).

As previously described, meiosis involving two circular *rad3*Δ*tel1*Δ strains yields complete inviability; no colonies form from 300 plated (Figure 3.2 B)(Naito et al., 1998). This slight difference in viability between meiosis involving two circular *trt1*Δ strains and two circular *rad3*Δ*tel1*Δ strains may be due to lack of the Rad3 checkpoint protein. Rad3 is required for the meiotic checkpoint, allowing a delay for repair of DNA double strand breaks caused during meiotic recombination. Failure of cells to activate this checkpoint leads to defective meiosis and low spore viability. (Murakami and Nurse, 1999; Shimada et al., 2002). The difference in meiotic viability in *rad3*Δ*tel1*Δ circular strains compared with circular *trt1*Δ strains is likely to reflect a combine loss of viability from the lack of checkpoint function and the general poor meiosis of strains with circular chromosomes.

A possible cause of the problems occurring during meiosis in the circular strains is the topology of the chromosomes. Following meiotic homologous recombination in strains harbouring circular chromosomes, dicentric chromosomes may form, causing chromosome breakage upon segregation of centromeres to opposite poles. The presence of a functional checkpoint pathway in circular *trt1*Δ strains may again allow the slightly higher percentage of survival compared with in the *rad3*Δ*tel1*Δ circular strains. It is also possible

A



Linear x linear | Circular *trt1*Δ x linear | Circular *pot1*Δ x linear | Circular *rad3*Δ*tel1*Δ x Circular *rad3*Δ*tel1*Δ

B

wild type linear x wild type linear	Circular *trt1*Δ x wild type linear	Circular *pot1*Δ x wild type linear	Circular *trt1*Δ x Circular *trt1*Δ	Circular *rad3*Δ*tel1*Δ h^{90}
97.3%	15%	17.7%	6.3%	0%

Figure 3.2 Strains with circular chromosomes are defective in meiosis

(A) DAPI staining of asci from meiosis involving circular chromosomes. Asci often have aberrant spores and contain fewer or more than than the normal four spores.
(B) Spore viability from meiosis involving circular chromosomes. 300 spores were plated in triplicate on YE4S and allowed to form colonies. Numbers are expressed as percentage of colonies formed compared with number of spores plated.

that the meiotic defects seen in circular strains is due to lack of telomeres and/or binding sites for telomere associated proteins. The meiotic behaviour of telomeres is very striking, with telomeres clustering in the meiotic prophase and the telomere led 'horsetail movement' of the nucleus (Chikashige et al., 1994). Loss of Taz1 in fission yeast causes disruption of meiotic telomere clustering and an aberrant horsetail movement. In a similar manner to circular strains lacking telomeres, meiosis involving telomeres lacking Taz1 is defective, leading to abberrant asci and low spore viability (Cooper et al., 1998).

3.4 Circular strains are sensitive to MMS

While circular strains are viable, they clearly display many problems. An interesting phenotype we observed is that strains with circular chromosomes are highly sensitive to DNA damage (Figure 3.3). When grown on plates containing low concentrations of the alkylating agent, methylmethane sulfonate (MMS) (0.001%), the circular strains show even greater sensitivity than *rad3*Δ cells, which lack both the intra-S-phase and DNA damage checkpoint pathways (Figure 3.3).

This extreme sensitivity could imply a role for telomeres in survival of damage, an idea supported by the mild MMS sensitivity seen in strains lacking the telomere binding protein Taz1 (Miller and Cooper, 2003), (Figure 3.3 A). Indeed, deletion of *taz1*$^+$ in the circular *trt1*Δ background has no effect on the sensitivity of these circular strains, indicating that the Taz1 effect stems from its role at telomeres (Figure 3.3 A) and again highlighting a potential role for functional telomeres in MMS resistance. Alternatively or in addition, the altered chromosomal topology of the circular strains may confer MMS hypersensitivity. Undergoing repair processes with a circular genome could conceivably result in dicentric chromosomes following recombinational DNA repair.

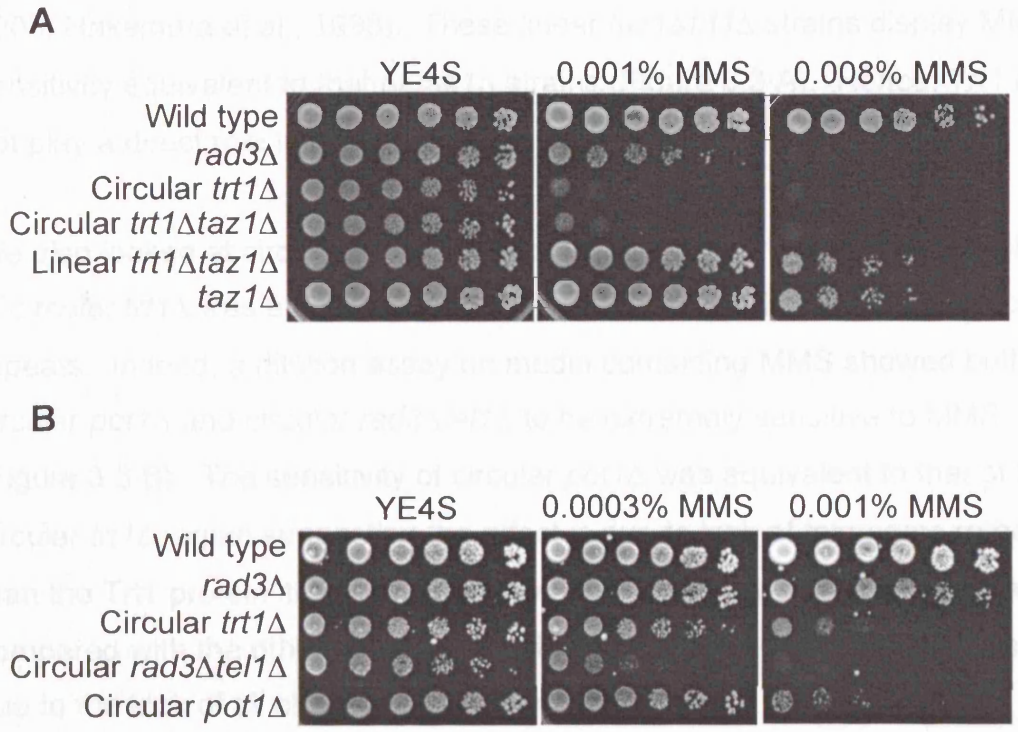


Figure 3.3 Circular *trt1*Δ strains are sensitive to DNA damage

(A) 5-fold serial dilution assay on plates containing MMS at the indicated levels. Circular *trt1*Δ cells display extreme sensitivity when grown on plates containing MMS. The sensitivity is is not affected by the presence of Taz1, but is dependent on loss of telomere sequences and circularisation of chromosomes. Circular strains are more sensitive than strains lacking the Rad3 checkpoint.
(B) Sensitivity to MMS is a general sensitivity of circular strains lacking telomeres as observed with circular *trt1*Δ, *pot1*Δ and *rad3*Δ *tel1*Δ strains.

Another potential explanation for the sensitivity of circular *trt1*Δ strains is that telomerase plays a direct role in DNA repair. Deletion of *trt1*$^{+}$ in a *taz1*Δ background leads primarily to survivors with linear chromosomes that maintain telomeres through a recombination-based method (Miller et al., 2006; Nakamura et al., 1998). These linear *taz1*Δ*trt1*Δ strains display MMS sensitivity equivalent to that of *taz1*Δ strains (Figure 3.3 A). Hence, Trt1 does not play a direct role in survival following DNA damage.

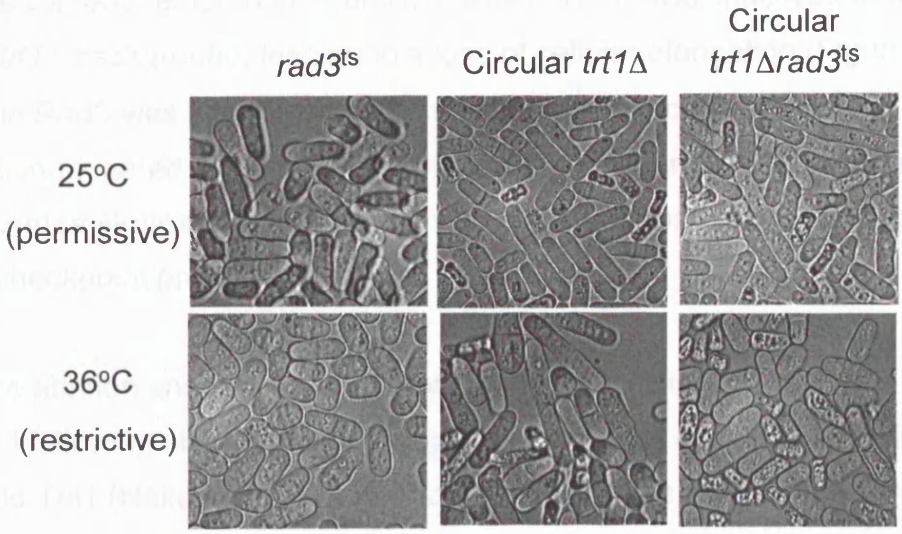
We also looked at circular strains with other genotypes to see if the sensitivity of circular *trt1*Δ was a general sensitivity of circular strains lacking telomere repeats. Indeed, a dilution assay on media containing MMS showed both circular *pot1*Δ and circular *rad3*Δ*tel1*Δ to be extremely sensitive to MMS (Figure 3.3 B). The sensitivity of circular *pot1*Δ was equivalent to that of the circular *trt1*Δ, again suggesting the effect is due to lack of telomeres rather than the Trt1 protein itself. The sensitivity of circular *rad3*Δ*tel1*Δ is increased compared with the other mutants containing circular chromosomes. This is due to the lack of all checkpoint function.

Thus, the sensitivity of the circular strains is due to a lack of telomeres rather than loss of the actual proteins, be it through the topological nature of the chromosomes or the absence of functional telomere repeats.

3.4.1 Circular *trt1*Δ strains activate a Rad3 dependent checkpoint

Following induction of DNA damage, checkpoint pathways are activated, allowing time for repair of damage before continuation of the mitotic cycle. Failure of a cell to activate the checkpoint would lead to mitosis in the presence of damage and cell death. Rad3, the ATR homolog in fission yeast is key to initiating both replication and damage checkpoints. Specificity is determined by phosphorylation of either of the effector kinases, Chk1 (G2/M damage checkpoint) or Cds1 (Intra-S phase replication checkpoint). As described earlier (Chapter 3.2), strains with circular chromosomes display an elongated phenotype, suggestive of checkpoint activation, even in the absence of genotoxic insult. However, many dead cells are also observed,

A



B

	Wild type	Circular *trt1Δ*	*rad3ts*	Circular *trt1Δrad3ts*	Circular *rad3Δtel1Δ*
25°C (permissive)	95%	58%	92%	65%	28%
36°C (restrictive)	91%	61%	89%	32%	24%

Figure 3.4 Circular *trt1Δ* strains activate a Rad3 dependent checkpoint

(A) Circular *trt1Δ* strains harbouring a *rad3ts* allele no longer elongate when grown at the restrictive temperature. An increase in number of dead cells is observed.
(B) Following loss of Rad3 checkpoint function, circular strains lose viability. Cultures were grown to log phase, 500 cells plated and incubated for 4 days. Colonies were counted and displayed as a percentage of the number of colonies formed compared with the number of cells plated.

primarily dying as non-elongated, partially divided cells (a cut phenotype), suggesting a defect in chromosome segregation without checkpoint activation. To look at the requirements of checkpoint activation in circular *trt1*Δ strains, we utilised a *rad3* temperature sensitive allele. Rad3 was inactivated in a circular *trt1*Δ background, leading to a loss of cellular elongation (Figure 3.4 A). While Rad3 was not essential for the survival of circular strains, its inactivation triggered elevated levels of cell death (Figure 3.4 B) in a manner reminiscent of strains having survived with circular chromosomes through loss of both checkpoint proteins, Rad3 and Tel1.

Telomere attrition and survival by chromosome circularisation are also observed following simultaneous disruption of the ATR and ATM homologues, Rad3 and Tel1 (Naito et al., 1998). *rad3*Δ*tel1*Δ cultures display many dead cells and, not surprisingly, the cells are not elongated (Figure 3.1 D). Circular *rad3*Δ*tel1*Δ strains display a greater sensitivity to DNA damaging agents than circular *trt1*Δ strains (Figure 3.3 B, Figure 3.5 A). Likewise, while circular *trt1*Δ*rad3*^{ts} cells display a sensitivity equivalent to that of circular *trt1*Δ when grown at the permissive temperature (Figure 3.5 A), the sensitivity increases to that of circular *rad3*Δ*tel1*Δ strains at the non-permissive temperature (Figure 3.5 A). Furthermore, following growth in the presence of MMS, circular *trt1*Δ cells show further elongation, indicative of checkpoint activation (Figure 3.5 B).

Thus, circular *trt1*Δ cells retain the Rad3 checkpoint function, and Rad3 plays a role in both the general viability of circular strains and promoting recovery from low levels of DNA damage.

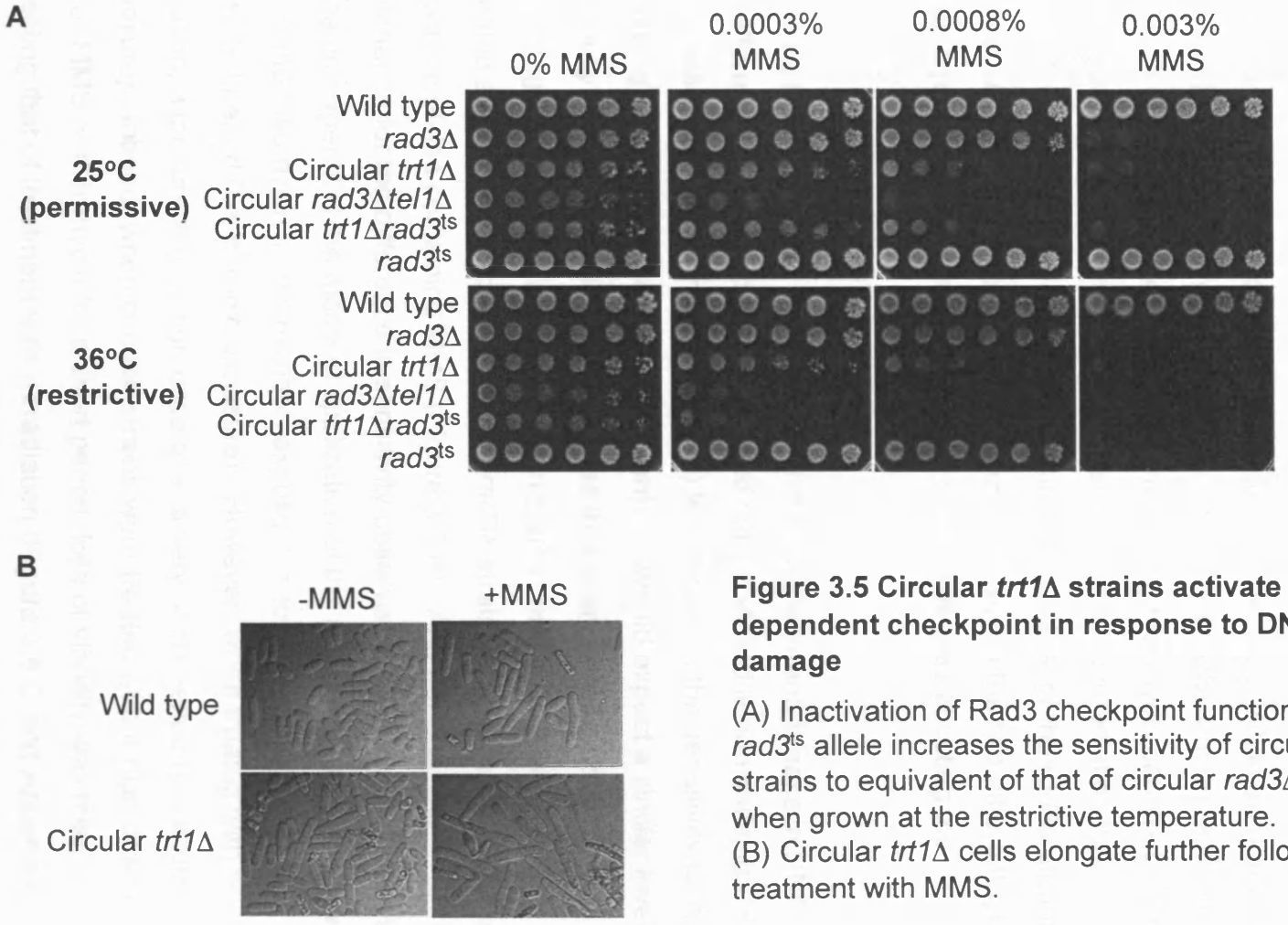


Figure 3.5 Circular *trt1*Δ strains activate a Rad3 dependent checkpoint in response to DNA damage

(A) Inactivation of Rad3 checkpoint function using a *rad3*^{ts} allele increases the sensitivity of circular *trt1*Δ strains to equivalent of that of circular *rad3*Δ*tel1*Δ when grown at the restrictive temperature.
(B) Circular *trt1*Δ cells elongate further following treatment with MMS.

3.4.2 Sensitivity of circular *trt1Δ* strains to damage is less pronounced following acute exposure

To further explore the conditions that challenge the viability of circular strains, we tested the sensitivity to a range of damaging agents. While alkylation of DNA by MMS creates single-strand lesions that form DSBs upon replication, exposure to γ-irradiation or the radiomimetic drug bleomycin causes DSBs immediately. While the circular strains are sensitive to γ-radiation the sensitivity is minimal and far less pronounced than that of the *rad3Δ* strains (Figure 3.6 A). This marked difference in sensitivity to the two different types of damage led us to question if the type of damage was affecting the sensitivity of the strains.

The drug bleomycin also causes immediate double strand breaks in DNA. If the difference in type of damage incurred (single strand lesion causing DSB upon replication versus immediate DSB) is reflected in the sensitivity to the damaging agent (MMS versus γ-radiation), we would expect a similar level of sensitivity when exposed to bleomycin as that seen with γ-irradiation. Surprisingly, growth of circular strains on plates containing bleomycin revealed a sensitivity exceeding that of *rad3Δ* strains in a manner similar to growth on plates containing MMS (Figure 3.6 B). Another possible explanation for the difference in sensitivity observed when exposed to these damaging agents is the mode of application of the agent. When treated with MMS and bleomycin in this manner, exposure is to a low dose for an extended period (i.e. chronic exposure). However, when treated with γ-radiation, exposure is to a high dose over a very short period (i.e. acute exposure). Indeed, when circular strains were treated with a high dose of either MMS or bleomycin for a short period, loss of viability was mild, reflecting that of treatment with γ-irradiation (Figure 3.6 C and Appendix Figure A1). This suggests that circular strains are particularly sensitive to any chronic induction of DSBs but only mildly sensitive to acute DSB induction. The mild sensitivity of circular strains following acute compared with chronic exposure to damage further demonstrates the ability of cells to activate a

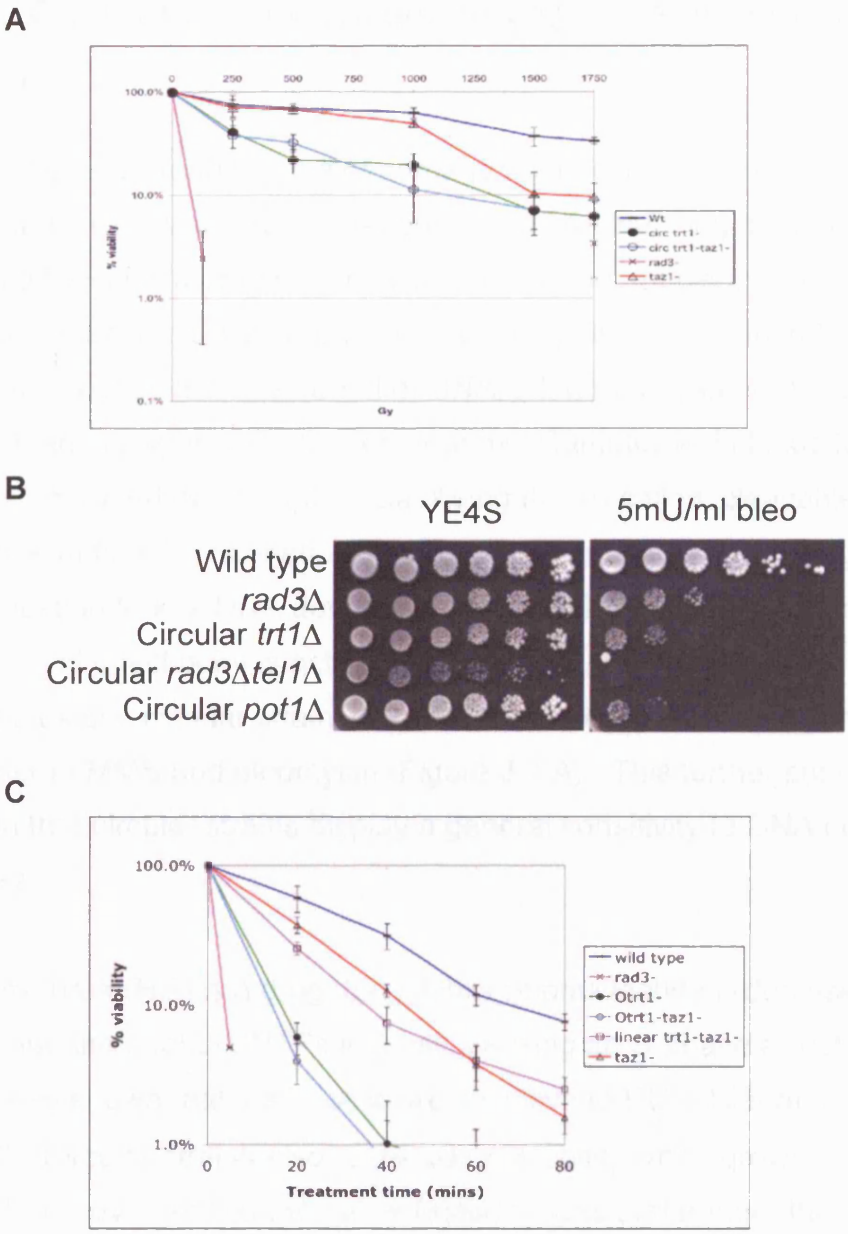


Figure 3.6 Sensitivity of Circular *trt1*Δ strain to DNA damage varies with chronic and acute exposure

(A) Exposure of circular *trt1*Δ strains to γ-radiation leads to loss of viability, but less than that of the *rad3*Δ checkpoint mutant.
(B) Chronic treatment of circular *trt1*Δ strains to the radiomimetic drug bleomycin causes extreme loss of viability. Sensitivity is greater than that of the *rad3*Δ checkpoint mutant.
(C) Acute treatment of circular *trt1*Δ strains with MMS causes a mild loss in viability. Sensitivity is less than that of the *rad3*Δ mutant.

checkpoint in response to damage; mutants lacking checkpoint function are highly damage sensitive, irrespective of the mode of application of treatment.

3.4.3 Circular strains are sensitive to a range of damaging agents

To further explore the range of damage the circular strains are sensitive to, we treated the strains with a variety of damage inducing agents. Camptothecin is a drug that inhibits the enzyme, topoisomerase I. Topoisomerase I is involved in alleviating topological strain in DNA. The enzyme cleaves a single strand of DNA, allowing passage of the second strand, and re-ligates the broken strands. Camptothecin binds the topoisomerase-DNA complex, stabilising the so-called 'cleavable complex', and preventing the re-ligation step following cleavage. Following passage of a replication fork, a DNA double strand break is formed and a Chk1 dependent checkpoint is activated (Wan et al., 1999). Following exposure to camptothecin, circular strains showed a severe loss of viability in a similar manner to MMS and bleomycin (Figure 3.7 A). This further supports the notion that circular strains display a general sensitivity to DNA double strand breaks.

Hydroxyurea (HU) is a drug that inhibits ribonucleotide reductase, thereby depleting the pool of dNTPs in a cell, causing an S-phase arrest. Previous work has shown that *taz1*Δ cells are sensitive to HU (Miller and Cooper, 2003). Circular strains also show loss of viability when grown in the presence of HU, however, unlike with other tested modes of damage, the sensitivity is in fact less than that of the *rad3*Δ checkpoint mutant (Figure 3.7 B). This suggests that the severe sensitivity of circular strains is more specific to DNA damage rather than replication fork stalling.

As described earlier (Chapter 1.2), circular strains show DAPI staining patterns suggestive of chromosome segregation defects. We decided to address this further by looking at the sensitivity of the mutants to thiabendazole (TBZ), a microtubule depolymerising drug. Indeed, strains with circular chromosomes lacking telomere repeats are sensitive to TBZ (Figure 3.7 C). TBZ sensitivity could indicate an inability to activate the spindle

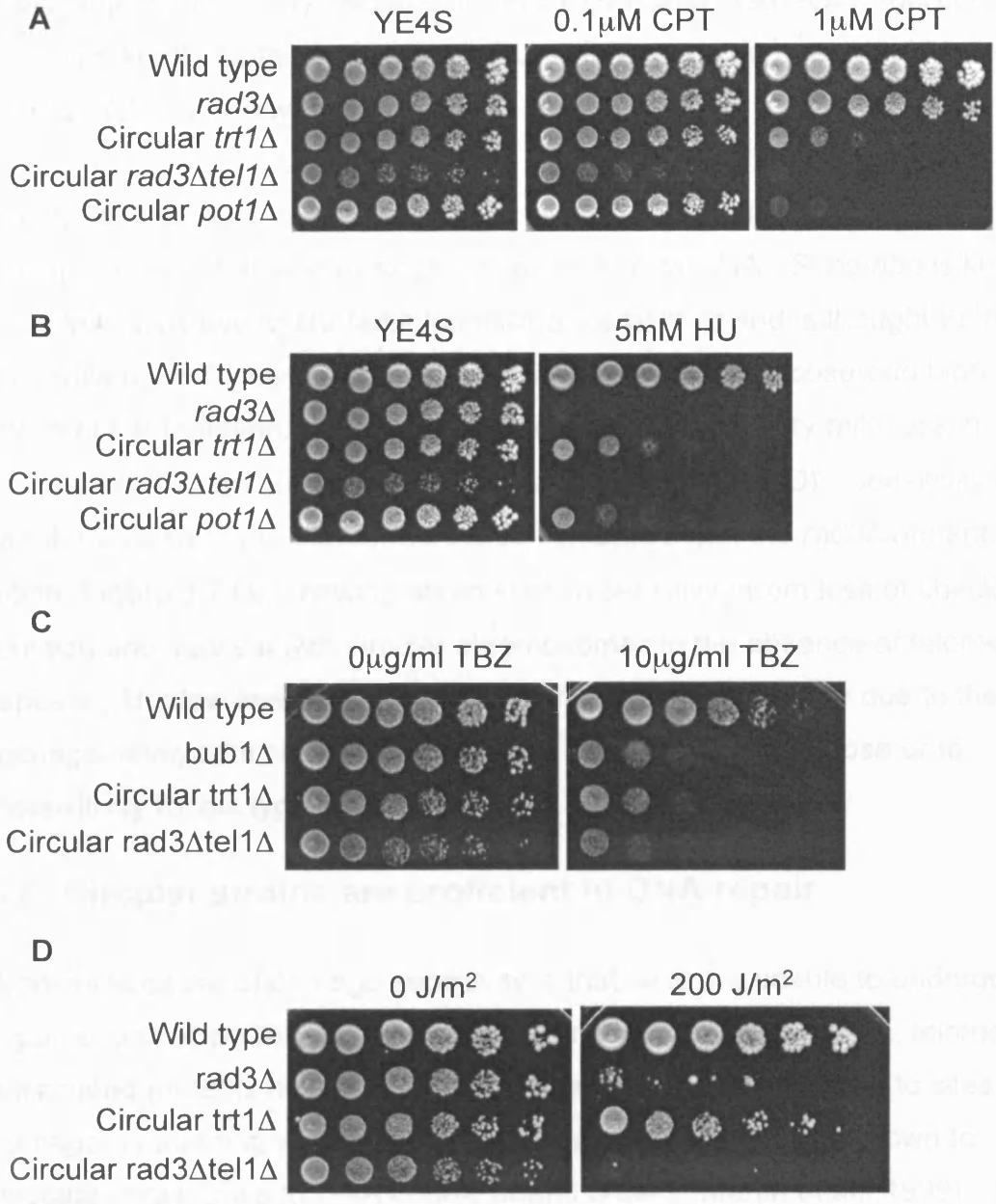


Figure 3.7. Circular *trt1*Δ strains are sensitive to a range of damaging agents

Five fold serial dilution assay on plates containing indicated drugs. Circular strains are sensitive to camptothecin (A), hydroxyurea (B), thiobendazole (C) and mildly sensitive to UV radiation (D).

assembly checkpoint (SAC) or problems with microtubule dynamics. We were able to confirm that Bub1 and Mad2, components of the spindle assembly checkpoint, do localise in circular strains, suggesting they have an active spindle assembly checkpoint. Bub1-GFP and Mad2-GFP foci can be observed in exponentially growing cultures to a same degree as wild type strains (data not shown).

Finally, we looked at the sensitivity of cells to UV radiation. UV causes thymine dimers that lead to single strand lesions in DNA. *S. pombe* is known to be less sensitive to UV radiation than *S. cerevisiae* and is thought to have an additional pathway to repair UV damage. Following exposure to high levels of UV radiation, circular *trt1*Δ strains show only a very mild loss in viability when compared with wild type strains (Figure 3.7 D). Sensitivity of circular *rad3*Δ*tel1*Δ is slightly increased compared with the *rad3*Δ mutant alone (Figure 3.7 D), showing an addition in sensitivity from loss of checkpoint function and survival with circular chromosomes in the absence of telomere repeats. The low level of sensitivity to UV-radiation is possibly due to the damage being administered as an acute rather than chronic dose or to insensitivity to this type of damage.

3.5 Circular strains are proficient in DNA repair

A possible cause of damage sensitivity is that cells are unable to undergo repair of damaged chromosomes. In other experimental systems, telomere associated proteins have been shown to relocate from telomeres to sites of damage. In budding yeast, the Ku and Sir proteins have been shown to relocate in response to DNA double strand breaks (Martin et al., 1999). The human telomere binding protein TRF2 has been shown to be phosphorylated in an ATM dependent manner in response to DNA damage and migrates very transiently to sites of damage (Bradshaw et al., 2005; Tanaka et al., 2005). It is possible that telomeres are required to act as a sink for repair proteins, stabilising them within the cell, so they are able to relocate in response to damage. In the absence of telomere repeats, proteins involved in DNA repair may become unstable and therefore less efficient at responding to DNA damage.

To address the possibility that circular strains lacking telomeres are unable to undergo efficient repair of broken chromosomes, we carried out a repair assay, utilising pulsed field gel electrophoresis (PFGE). Following a low dose of damage by γ-irradiation (100 Gy), chromosomes are sufficiently damaged that by whole chromosome pulsed field gel analysis, most of the intact chromosomes are no longer observed; the broken DNA running as a low molecular weight smear (Figure 3.8 A). Sampling cultures in the hours following damage, repair of the DNA can be observed as intact chromosomes appear in the gel and the smear of broken DNA disappears (Figure 3.8 A). Carrying out this assay on strains with circular chromosomes poses problems; the circular chromosomes do not enter a pulsed field gel when intact. However, disappearance of the smear of broken DNA may provide an assessment of repair. Indeed, following damage of strains with circular chromosomes, disappearance of the low molecular weight smear can be observed in this manner, suggesting that repair is occurring (Figure 3.8).

A caveat to this experiment is that we treat the cells with an acute dose of damage. As described earlier, sensitivity is less severe when treated in this manner compared with a chronic dose. However, from this experiment we are able to observe that repair processes are functional in circular strains, suggesting an inability to repair DSBs is not the basis for the defect in circular strains. It is interesting to observe that, while repair does occur in circular *trt1*Δ strains, the efficiency and/or speed of repair may be less than in a wild type strain. This could contribute to the damage sensitivity of circular strains. It could also explain why, following chronic treatment with a damaging agent, the strains are particularly sensitive compared with an acute dose. The less efficient repair would mean that cells would accumulate DNA damage to a higher level than if repair was more efficient as in a wild type strain. This accumulation of damage may push the level past a limit that the cell it is able to cope with, causing cell death. However, due to the limits of the experimental system, we were unable to address this issue fully.

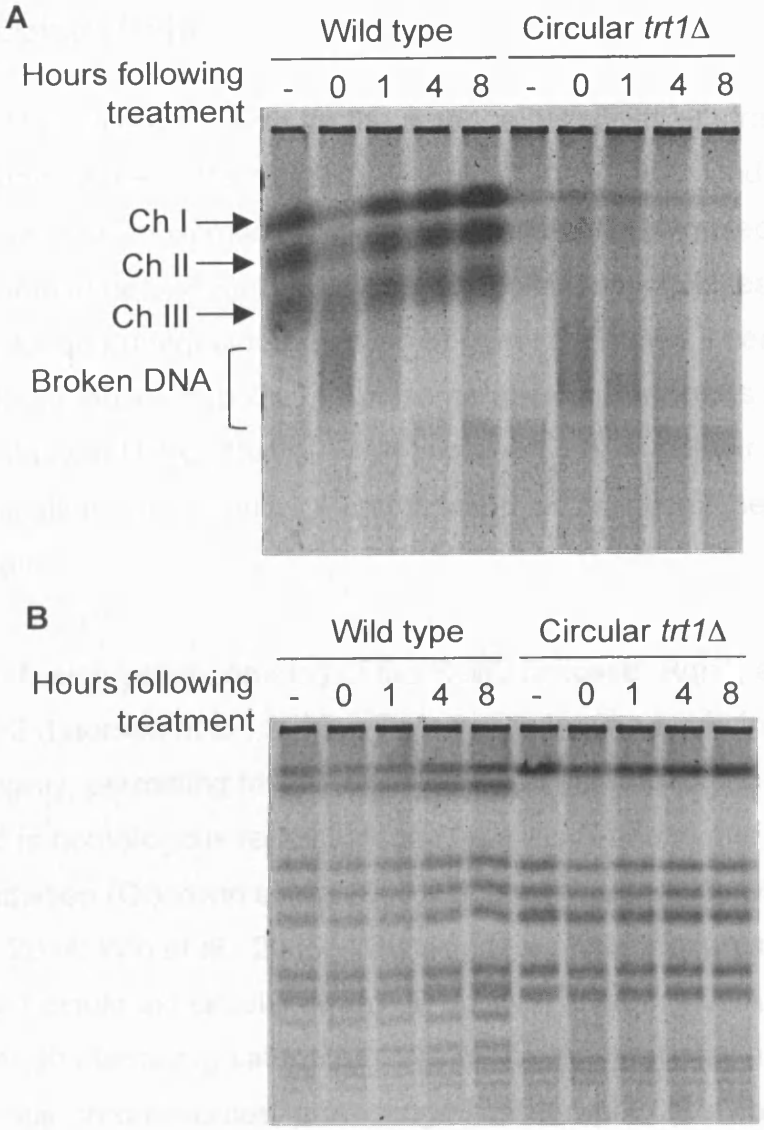


Figure 3.8 Circular strains are proficient in DNA repair

(A) Whole chromosome pulsed field gel analysis allows repair of broken chromosomes to be observed in the hours following exposure to 100 Gy γ-radiation. Following treatment, cells were allowed to recover at 32°C and samples processed for PFGE at the indicated time points. Repair of chromosomes can be seen in wild type strains as reappearance of chromosome bands in the gel and disappearance of the smear of broken DNA during recovery. In circular *trt1*Δ strains, disappearance of the smear of broken DNA can be observed, reflecting repair of the damage.
(B) NotI digest of plugs used in (A) to show equal loading of DNA.

3.6 Over expression of topoisomerase III, Rqh1 helicase or topoisomerase II does not affect damage sensitivity of circular strains

Another potential cause for the damage sensitivity of strains with circular chromosomes is the topology of the chromosomes. Undergoing repair with circular chromosomes may lead to catenated DNA molecules and dicentric chromosomes. Segregation of such molecules would lead to further DNA breaks and unequal segregation of genetic material if centromeres were pulled to opposite poles. Topoisomerases are enzymes that modify the topology of DNA. They play an important role in cellular processes from replication, transcription, recombination, chromosome segregation and DNA repair.

The fission yeast homolog of the RecQ helicase, Rqh1, acts in concert with Top3 (Laursen et al., 2003). Together they play a role in maintaining genome integrity, permitting faithful segregation of chromosomes following replication and in homologous recombination repair in S-phase and G2 following UV irradiation (Goodwin et al., 1999; Laursen et al., 2003; Oh et al., 2002; Win et al., 2004; Win et al., 2005). We wondered if over expression of either Top3 or Rqh1 would aid circular strains in survival following DNA damage, either through alleviating catenated DNA molecules that may arise from having circular chromosomes, promoting efficient repair, or a combination of both. We found that over expression of Top3 or Rqh1 did not aid in the survival of circular strains following DNA damage (Figure 3.9 A).

Topoisomerase II is required for the separation of mitotic chromosomes in *S. pombe* (Uemura et al., 1987). It has also previously been shown to aid in the segregation of a circular mini chromosome in fission yeast (Murakami et al., 1995). To see if Top2 could aid in the repair defects seen in strains with circular chromosomes, perhaps through resolving catenated DNA structures that may be formed following repair of circular chromosomes, we overexpressed Top2. In circular strains overexpressing Top2, we did not observe any increase in viability following treatment with MMS (Figure 3.9 B).

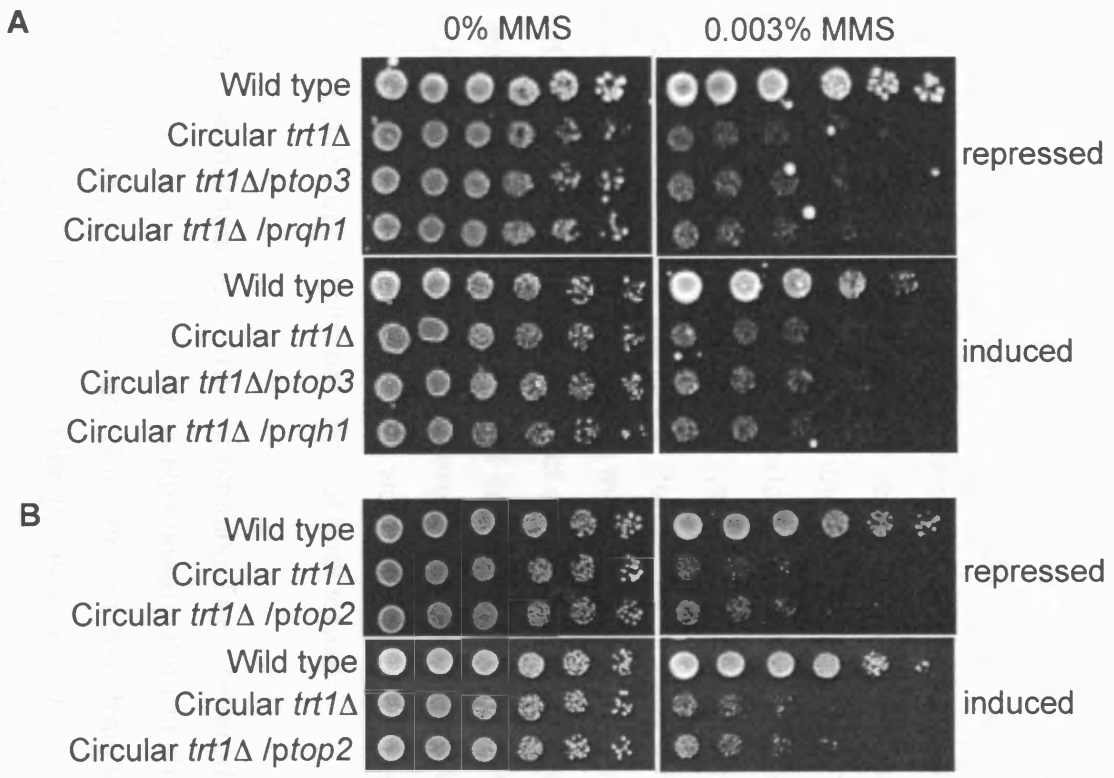


Figure 3.9 Over-expression of topoisomerases or Rqh1 helicase does not affect damage sensitivity of strains with circular chromosomes

Five fold serial dilution assay on minimal media containing the indicated amounts of MMS. Genes were expressed from plasmids on the thiamine repressible NMT promotor. The assay was carried out in the presence (repressed) and absence (induced) of thiamine. Overexpression of Top3, Rqh1 (A) or Top2 (B) does not affect the damage sensitivity of circular strains.

3.7 Reintroduction of telomerase to circular *trt1Δ* strains

We were intrigued to know the effect of reactivating telomerase in a circular strain with no ends to act on. Analysis by whole chromosome pulsed field gel electrophoresis distinguishes linear and circular chromosomes; linear chromosomes enter the gel and run as three individual entities, whereas the topology of circular chromosomes prevents entry into the gel (Figure 3.10 A, lanes 1 and 2). Surprisingly, reintroduction of *trt1*$^{+}$ to a circular *trt1Δ* strain allows entry of only chromosome III in a whole chromosome pulsed field gel, as seen by staining with ethidium bromide or by Southern blotting and hybridisation to rDNA repeats, specific for chromosome III (Figure 3.10 A and B). Hybridisation with a telomere repeat probe demonstrates that telomeres are added to the linearised chromosome III (Figure 3.10 C). The rDNA repeats are located just centromeric to the telomere repeats at either end of Chromosome III. The rDNA regions undergo constant recombination due to the repetitive nature of the DNA. Furthermore, 13bp stretches of telomere sequence are interspersed within the rDNA repeats (Sugawara, 1989). These short telomere sequences may act as telomere seeds, allowing engagement of telomerase when a broken end is generated by rDNA recombination reactions. The absence of telomere seeds and highly recombinogenic regions on chromosomes I and II would explain why these chromosomes remain closed upon reintroduction of telomerase.

In a similar manner to a strain containing Taz1, reintroduction of Trt1 to a circular *trt1Δtaz1Δ* strain also causes linearisation of chromosome III (Figure 3.10). Carrying out a telomere Southern on this strain we can observe a similar deregulation in telomere length as in a *taz1Δ* strain with three linear chromosomes (Figure 3.11).

Figure 3.10 Reintroduction of telomerase to circular *trt1*Δ strains causes linearisation of chromosome III

(A) Ethidium Bromide stained whole chromosome pulsed field gel shows entry of a single chromosome upon expression of *trt1*$^{+}$ in a strain having survived with circular chromosomes through disruption of telomerase.
(B) Probing the same gel for rDNA repeats demonstrates the chromosome entering the gel is chromosome III.
(C) Probing the same gel for telomere repeats demonstrates chromosome III has linearised through the addition of telomere repeats.

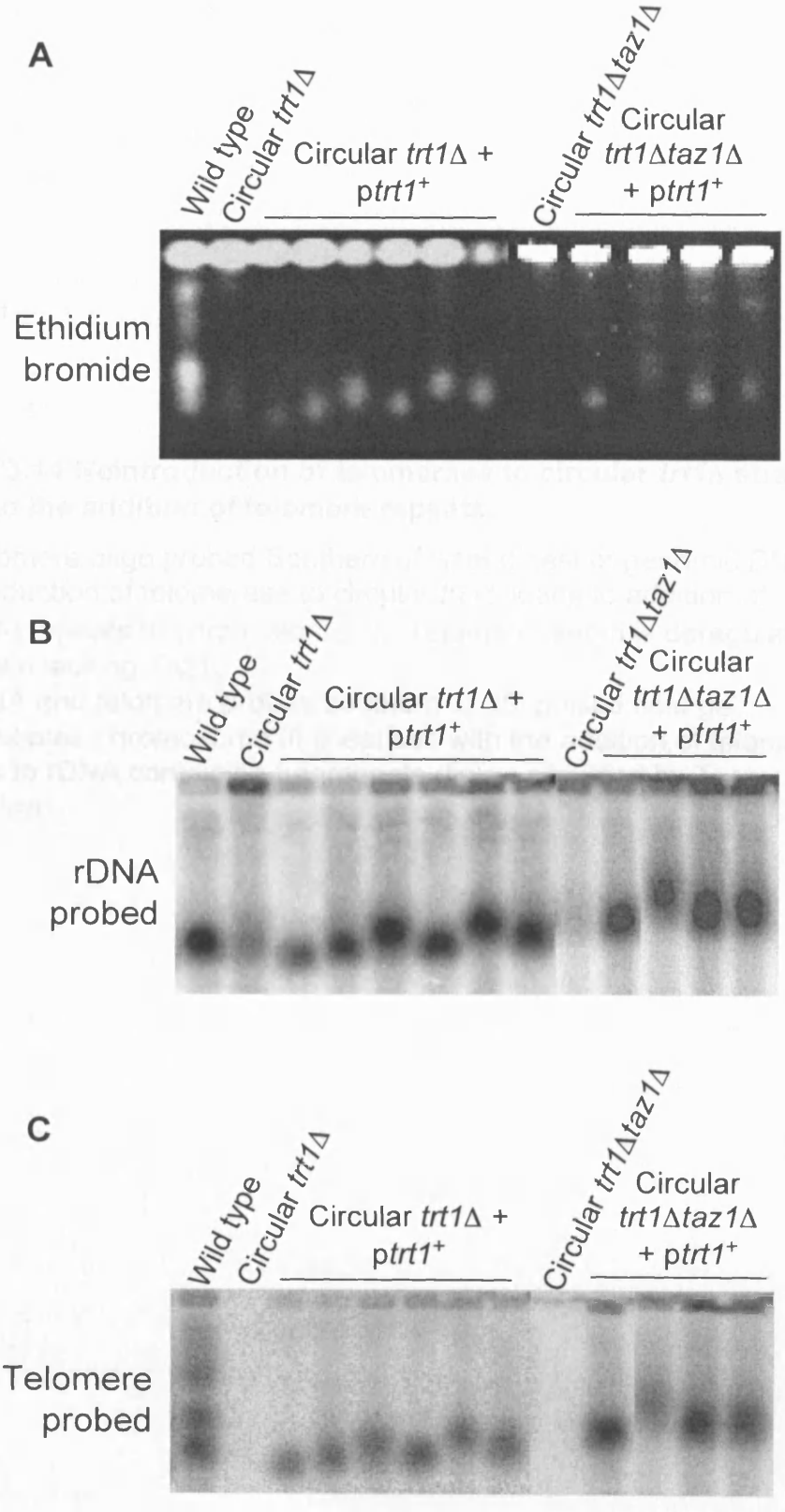
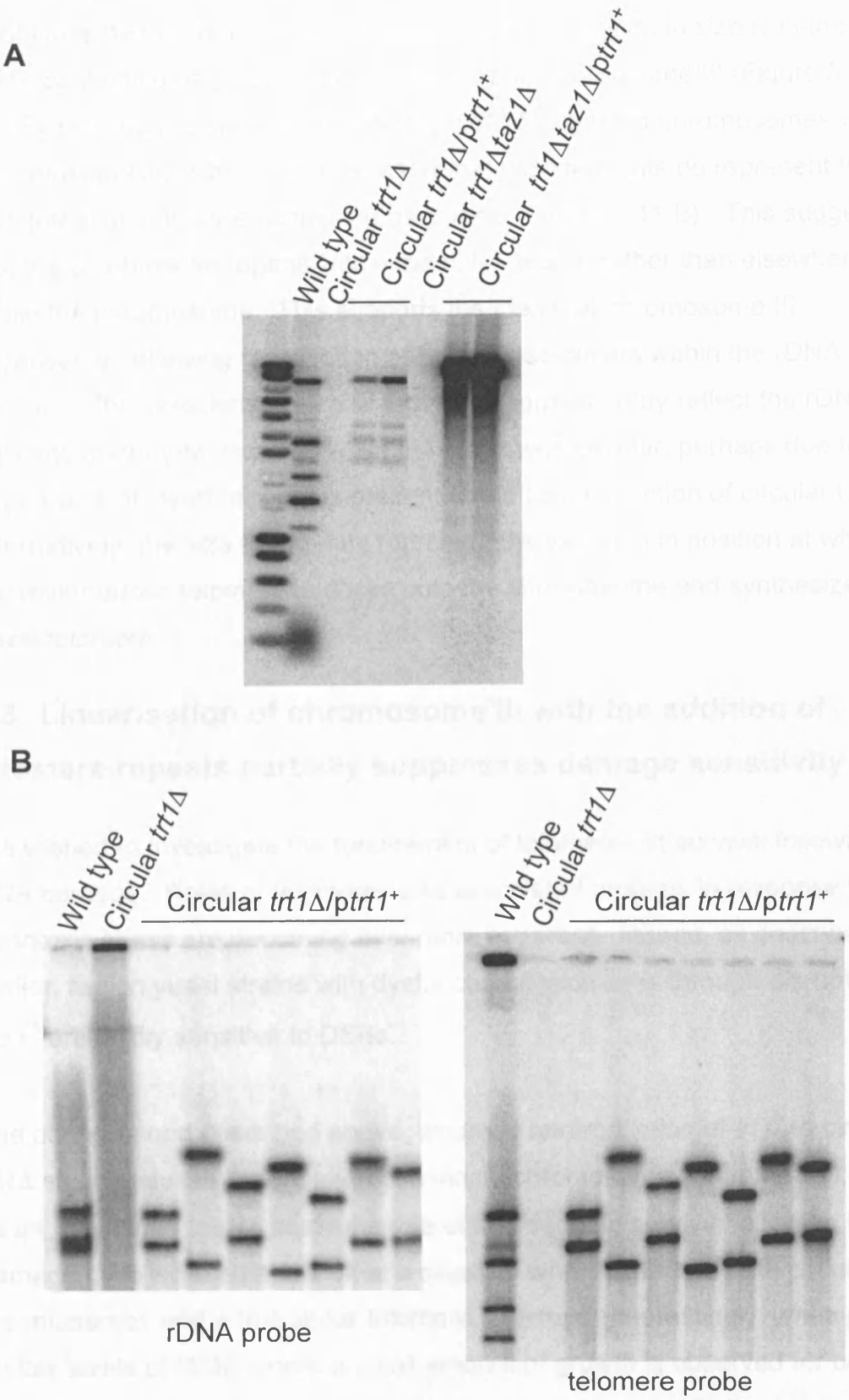

A
Wild type
Circular trt1Δ
Circular trt1Δ + ptrt1+
Circular trt1Δtaz1Δ
Circular trt1Δtaz1Δ + ptrt1+
Ethidium bromide
B
Wild type
Circular trt1Δ
Circular trt1Δ + ptrt1+
Circular trt1Δtaz1Δ
Circular trt1Δtaz1Δ + ptrt1+
rDNA probed
C
Wild type
Circular trt1Δ
Circular trt1Δ + ptrt1+
Circular trt1Δtaz1Δ
Circular trt1Δtaz1Δ + ptrt1+
Telomere probed

Figure 3.11 Reintroduction of telomerase to circular *trt1*Δ strains leads to the addition of telomere repeats

(A) Telomere oligo probed Southern of ApaI digest of genomic DNA. Reintroduction of telomerase to circular *trt1*Δ leads to addition of telomere repeats to chromosome III. Telomere length is deregulated in a strain lacking Taz1.
(B) rDNA and telomere probed southern of SfiI pulsed field gel demonstrates chromosome III linearises with the addition of telomere repeats to rDNA containing fragmenets (figure provided by T. Nakamura).


A
Wild type
Circular trt1Δ
Circular trt1Δ/ptrt1+
Circular trt1Δtaz1Δ
Circular trt1Δtaz1Δ/ptrt1+
B
Wild type
Circular trt1Δ
Circular trt1Δ/ptrt1+
rDNA probe
Wild type
Circular trt1Δ
Circular trt1Δ/ptrt1+
telomere probe

From the whole chromosome pulsed field gel we can see upon linearisation of chromosome III that it varies in size significantly between transformant (Figure 3.10). Inspection of specific fragments of chromosome III resolved by PFGE of SfiI digested chromosomes reveals this great variation in size is in the rDNA containing fragments upon reopening of chromosome III (Figure 3.11 B). By probing the same pulsed field gel of SfiI digested chromosomes with a telomere specific probe, it can be seen that the fragments do represent the new terminal, telomere containing fragments (Figure 3.11 B). This suggests that the chromosome opens within the rDNA region rather than elsewhere within the chromosome. This supports the idea that chromosome III linearisation following reactivation of telomerase occurs within the rDNA repeats. The variations in size of terminal fragments may reflect the rDNA rearrangement that occurred while the strain was circular, perhaps due to the large tracts of inverted repeats present at the fusion junction of circular Chr III. Alternatively, the size range may represent the variation in position at which the reintroduced telomerase docks onto the chromosome and synthesizes a novel telomere.

3.8 Linearisation of chromosome III with the addition of telomere repeats partially suppresses damage sensitivity

We wished to investigate the requirement of telomeres in survival following DNA damage. Roles of telomeres and associated proteins in response to genotoxic stress are becoming ever more apparent. Indeed, as described earlier, fission yeast strains with dysfunctional telomeres through disruption of *taz1*$^+$ are mildly sensitive to DSBs.

The phenomenon described above, whereby reintroduction of *trt1*$^+$ to circular *trt1*Δ strains causes linearisation of a single chromosome (Chapter 3.7), gave us an interesting tool to study the role of telomeres in survival following DNA damage. We were able to look at a situation where cells had both circular chromosomes and a true linear telomere structure. Interestingly, when grown on low levels of MMS where a small amount of growth is observed for both *rad3*Δ and circular *trt1*Δ, a partial suppression of drug sensitivity can easily be observed in circular strains expressing telomerase (Figure 3.12). The strains

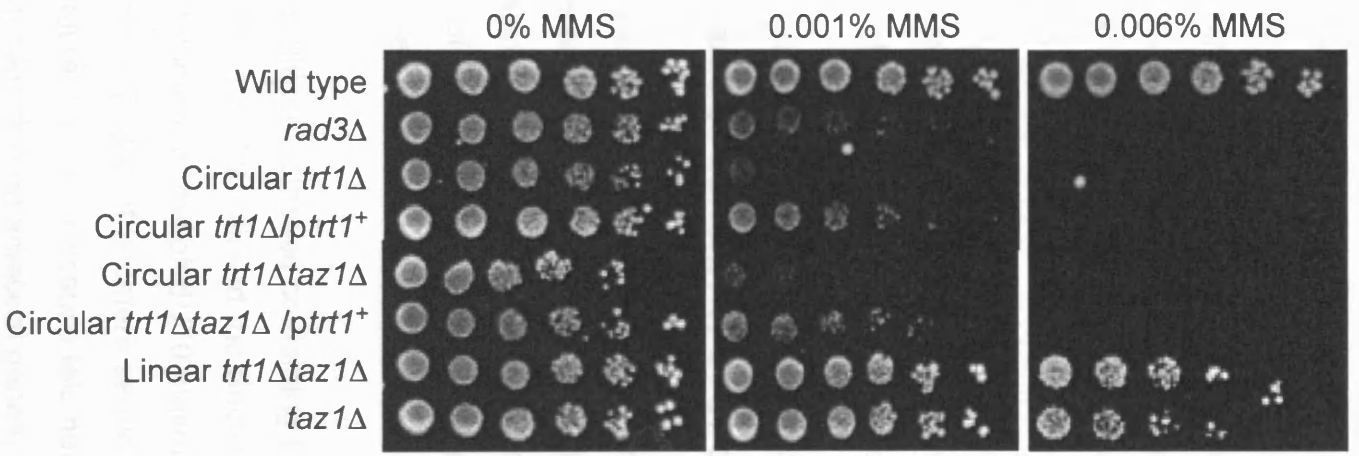


Figure 3.12 Linearisation of chromosome III with the addition of telomere repeats partially suppresses the damage sensitivity of circular *trt1*Δ strains

A 5-fold serial dilution of log phase cultures containing a multicopy Trt1 expression vector or empty vector. 5μl of each dilution was spotted onto rich media containing G418 and the indicated amounts of MMS. Strains with linearised chromosome III through re-expression of Trt1 display a partially suppressed sensitivity to MMS. The sensitivity was independent of the presence or absence of Taz1.

are still, however, very sensitive to high levels of MMS, to a level similar to the *rad3*Δ mutant. While the presence of functional telomeres within a cell clearly aids survival following genotoxic stress, we can not be sure if this partial rescue is due to the actual presence of telomere repeats or the reduction (by one third) in potential topological problems a cell may have to deal with when repairing circular chromosomes. Interestingly, despite the presence of deregulated telomere length upon reintroduction of telomerase to circular *trt1*Δ*taz1*Δ strains (Chapter 3.7, Figure 3.11), the same level of suppression of damage sensitivity is observed in a strain lacking Taz1 to a strain containing Taz1 (Figure 3.12). One might expect to see a slight increase in sensitivity in the strain lacking Taz1p when telomeres are present given the sensitivity of a *taz1*Δ mutant is due to dysfunctional telomeres. However, this might reflect the high level of MMS required to observe the *taz1*Δ defect, a level at which there is complete loss of viability of a circular strain.

3.9 The presence of a telomere containing plasmid does not affect the damage sensitivity of circular strains

Another way we thought to look at the requirement of telomeres following DNA damage was to transform a circular *trt1*Δ strain with a multicopy plasmid containing fission yeast telomere repeats. In this way we were able to look at the presence of many internally placed telomere sequences in a strain still containing three circular chromosomes.

We cloned a multicopy plasmid containing telomere repeats to express in *S. pombe* (Figure 3.13). We utilised the SacI-ApaI fragment from the cloned fission yeast telomere of the pNSU70 plasmid (Sugawara, 1989). This fragment contains 250bp of telomere sequence and only 32bp of STE sequence (Figure 3.13 A). Because telomere sequences confer transcriptional silencing on adjacent genes, we carried out the experiment on *rik1*Δ strains to allow for selection of *LEU2* expression and therefore retention of the plasmid. Rik1p is required for telomere silencing in fission yeast (Ekwall et al., 1996) but has no effect on damage sensitivity (data not shown). Retention of plasmid and telomere sequence within the plasmid was also

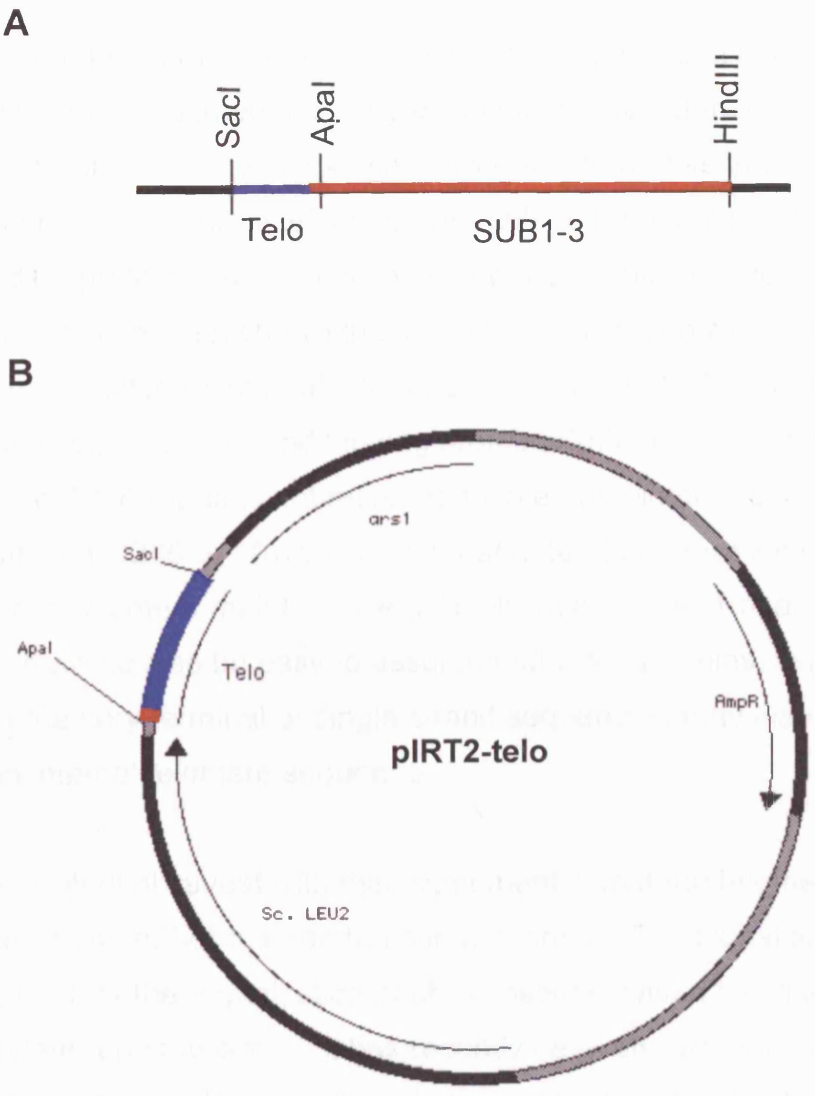


Figure 3.13 Multicopy telomere plasmid

(A) Restriction map of a cloned *S. pombe* telomere from pNSU70. The 250bp SacI-ApaI telomere fragment, containing 32bp sub-telomeric DNA, was cut out and cloned into pIRT2 for transformation into *S. pombe* (B).

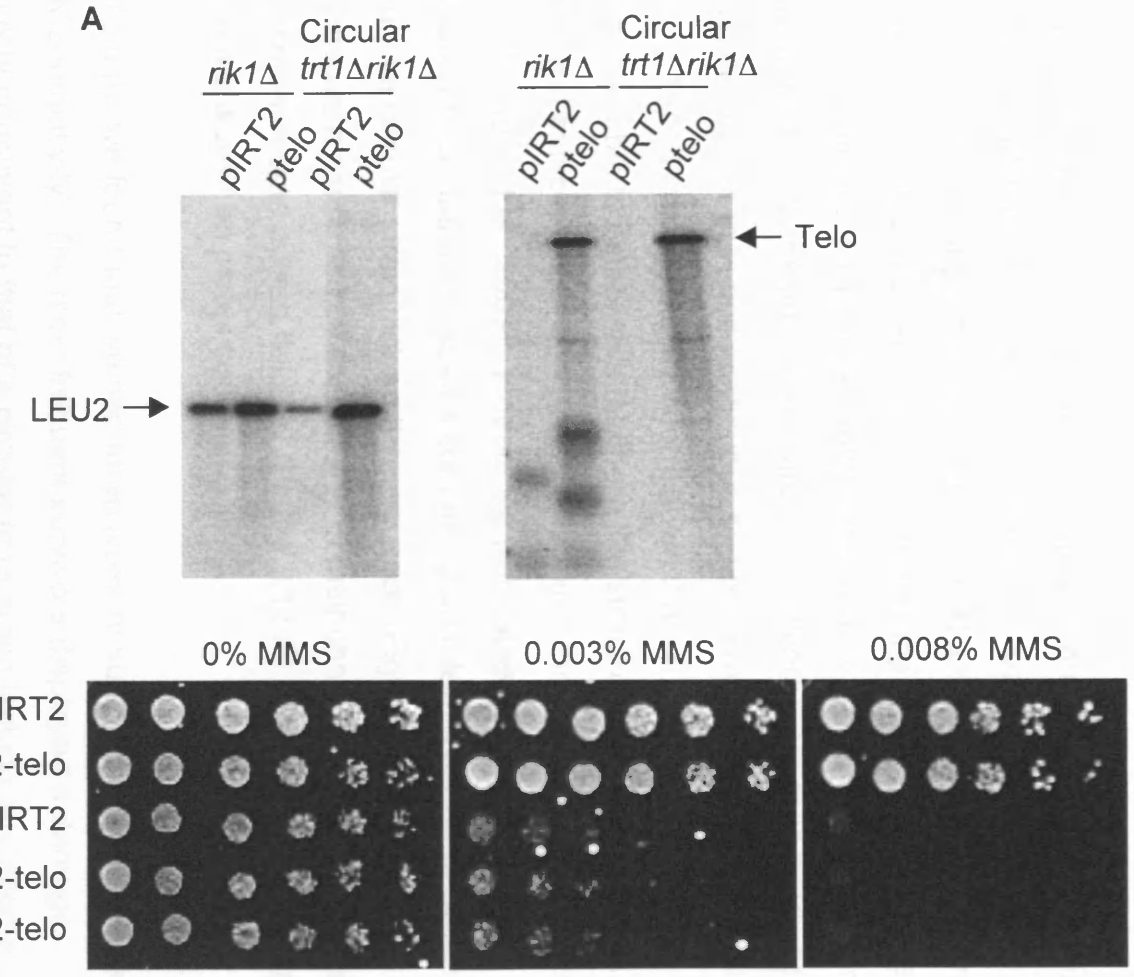
confirmed by southern analysis (Figure 3.14 A). Dilution assays on MMS showed that the presence of the telomere-containing plasmid was not sufficient to allow circular strains to survive following MMS treatment (Figure 3.14 B).

This experiment, however, has caveats. The telomere sequences are not 'true' telomeres; telomeres are, by definition, the structures at chromosome ends. Internal telomere sequences lack ends as well as the single stranded overhang. While internal telomeres are able to recruit at least a proportion of the binding proteins involved in setting up the telomere structure, such as Taz1, it has also been shown that not all the proteins present at chromosome ends are recruited to internal telomeres (Sadaie et al., 2003). Perhaps unsurprisingly, the DNA end binding protein, Ku70, involved in the NHEJ process of DNA repair is not recruited to internal telomere sequences (Miyoshi et al., 2003). Ku70 is present at natural chromosome ends and is involved in telomere maintenance (Manolis et al., 2001; Nakamura et al., 2002). It would also be easy to assume that other proteins, especially those binding the very terminal or single strand sequences, may also be absent from an internal telomere sequence.

Another potential caveat with this experiment is that the telomere is present as an episome rather than on the chromosomes. Functional telomeres may be required for the organisation of chromosomes within a cell to allow appropriate repair to occur. It has recently been shown that telomere clustering at the nuclear periphery is required for repair of subtelomeric regions in budding yeast (Therizols et al., 2006). While it was also shown that clustering at the periphery was not required for the repair of more internal breaks within the genome, this does not exclude the possibility of a requirement for clustering in general DNA repair in fission yeast. Alternatively, a similar, telomere dependent organisation of chromosomes not involving the nuclear periphery may be required for organising chromosomes for general repair of genomic DNA.

Figure 3.14 A multicopy telomere plasmid does not affect the damage sensitivity of circular *trt1*Δ strains

(A) Southern analysis demonstrates retention of plasmid and retention of telomere sequence within the plasmid. Southerns of NsiI digested genomic DNA probed with the *S. cerevisiae LEU2* gene and random primed telomere probe.
(B) The presence of many telomere sequences on a plasmid does not affect damage sensitivity of circular strains.


A
Circular
rik1Δ trt1Δrik1Δ
pIRT2
ptelo
pIRT2
ptelo
LEU2
Telo
B
0% MMS
0.003% MMS
0.008% MMS
rik1Δ/pIRT2
rik1Δ /pIRT2-telo
Circular trt1Δ rik1Δ/pIRT2
Circular trt1Δ rik1Δ /pIRT2-telo
Circular trt1Δ rik1Δ /pIRT2-telo

3.10 A single genomic telomere sequence is not sufficient to suppress damage sensitivity

To address the question of whether a genomic telomere sequence is sufficient to allow survival following damage, we wanted to look at the sensitivity of a circular strain containing a telomere sequence at a locus within the genome. We inserted a 250bp telomere sequence containing 32bp STE at the *ura4* locus of a linear *trt1*$^+$ strain, selecting for integration by negative selection on 5'-FOA (Figure 3.15, Figure 3.16). Circular survivors were then obtained through deletion of *trt1*$^+$ and continuous streaking on plates for single colonies (Figure 3.16). It has previously been shown that an internal telomere sequence is able to establish a telomere like structure through the recruitment of Taz1 and establishment of a transcriptionally repressive heterochromatin domain (Sadaie et al., 2003). Indeed, we were able to see Taz1-GFP localisation in circular *trt1*Δ strains containing an internal telomere sequence, but not in a circular strain lacking the sequence (Figure 3.16). Circular survivors were confirmed by the inability of their chromosomes to enter a whole chromosome pulsed field gel (Figure 3.17 A). Sensitivity to damage was then assayed on plates containing MMS.

Surprisingly, we found that we had three types of survivor with varying levels of MMS sensitivity. The most frequent survivors displayed a damage sensitivity equivalent to that of a circular *trt1*Δ strain lacking any telomere repeats, referred to hereafter as 'C1' (Figure 3.18). Southern analysis confirmed these survivors had retained a 250bp telomere sequence at the *ura4* locus (Figure 3.17 B). The other survivors, 'X1' and 'X2' displayed varying degrees of mild sensitivity and also retained the 250bp telomere sequence at *ura4* (Figure 3.17 B). The presence of the internal telomere sequence in sensitive survivors, C1, demonstrates the suppression in sensitivity cannot be attributed to retention of telomere sequence at this site. We carried out further analysis on the survivors with suppressed sensitivity to understand the nature of their survival and the reasoning behind the suppression, an issue that is the focus of Chapter 4.

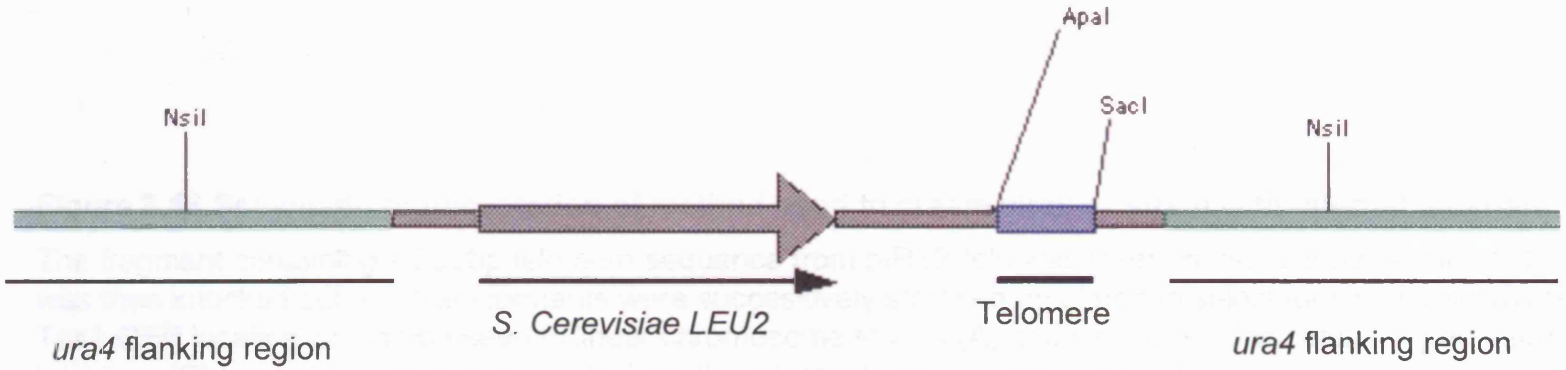


Figure 3.15 *ura4* locus of strain with internal telomere

A fragment containing the *S. cerevisiae LEU2* gene and 250bp telomere fragment amplified from pIRT2-Telo was integrated at the *ura4* locus. Selection for insertion was by loss of Ura4 expression on 5'-FOA.

Figure 3.16 Schematic representation of method used to create circular strain with internal telomere

The fragment containing a 250bp telomere sequence from pIRT2-telo was inserted into a linear strain. *trt1*$^{+}$ was then knocked out and transformants were successively streaked on plated to select for circular survivors. Taz1-GFP localisation can be seen in linear chromosome strains (A) and circular strains containing an internal telomere (C) but not in a circular strain lacking the internal telomere sequence (B).

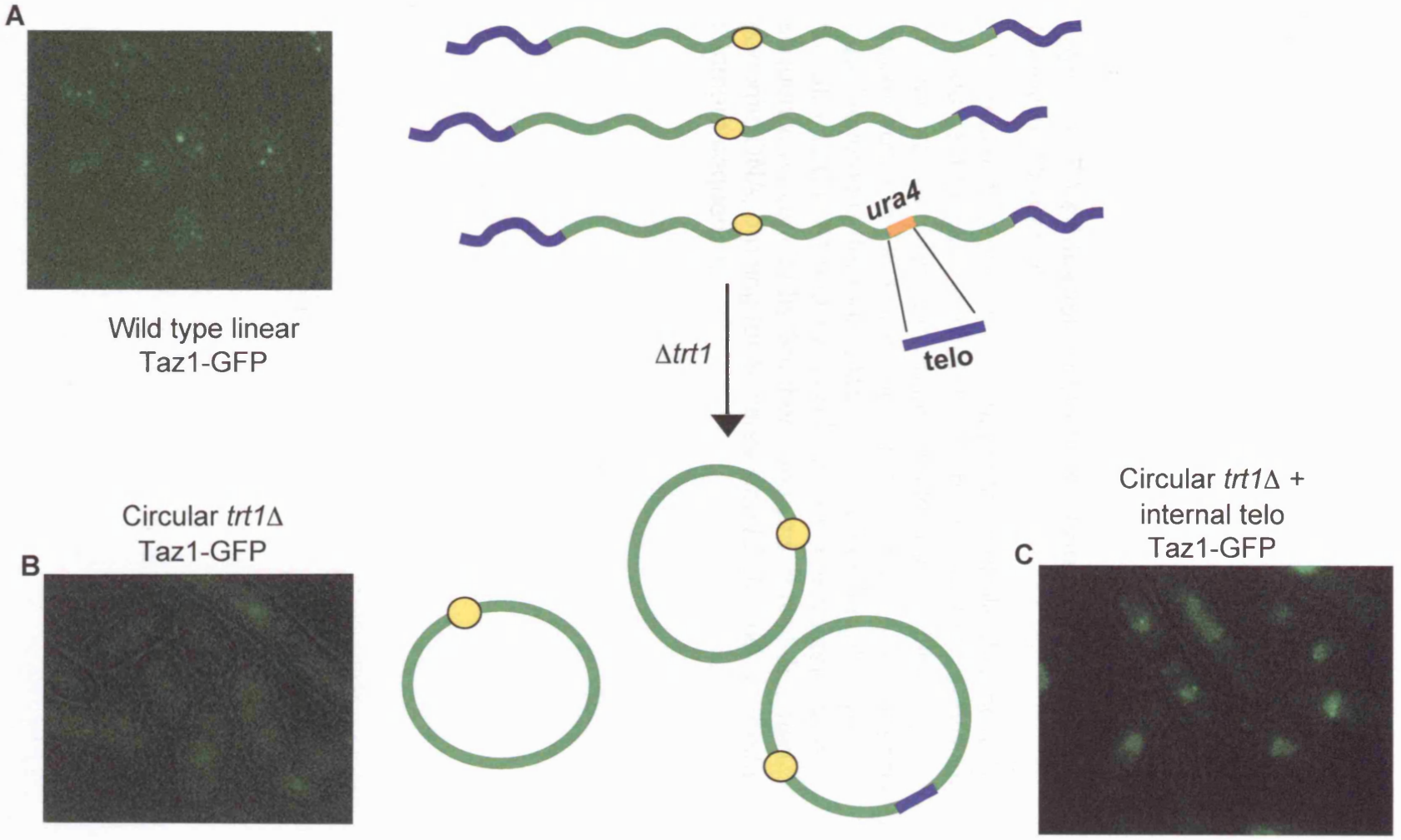
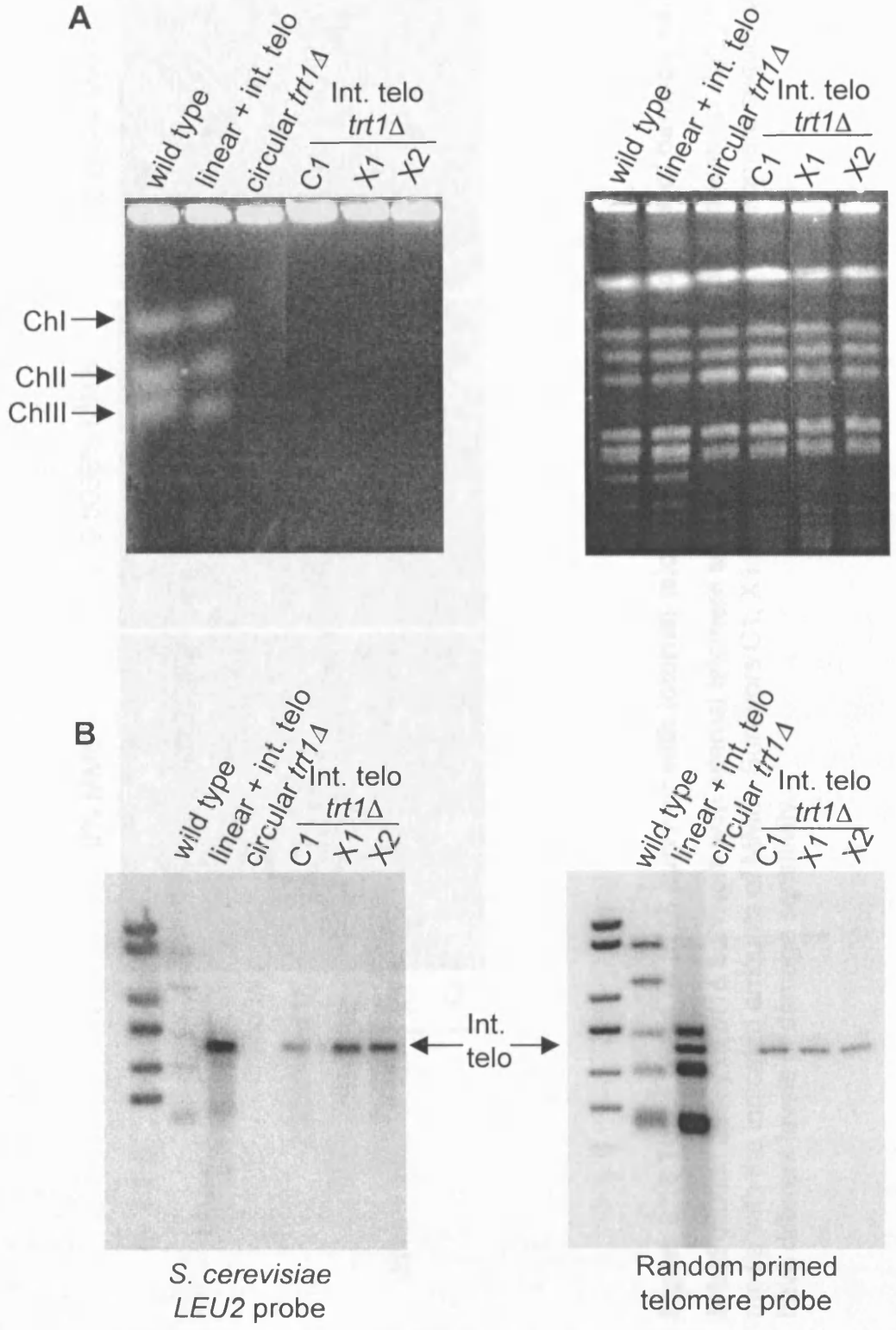

A
Wild type linear
Taz1-GFP
ura4
telo
Δtrt1
Circular trt1Δ
Taz1-GFP
B
Circular trt1Δ +
internal telo
Taz1-GFP
C

Figure 3.17 Verification of circular strains with internal telomere sequence

(A) Circular chromosomes do not enter a whole chromosome pulsed field gel. Survivors C1, X1 and X2 are *trt1*Δ survivors created from a strain with an internal telomere. Whole chromosome PFGE shows the chromosomes do not enter the gel. Equal loading is demonstrated by NotI digestion of plugs.
(B) Strains C1, X1 and X2 all contain the internal telomere sequence as verified by Southern analysis of NsiI digested genomic DNA, probing for *S. cerevisiae LEU2* and *S. pombe* telomere sequence.


A
wild type
linear + int. telo
circular trt1Δ
Int. telo
trt1Δ
C1
X1
X2
ChI
ChII
ChIII
B
Int.
telo
S. cerevisiae
LEU2 probe
Random primed
telomere probe

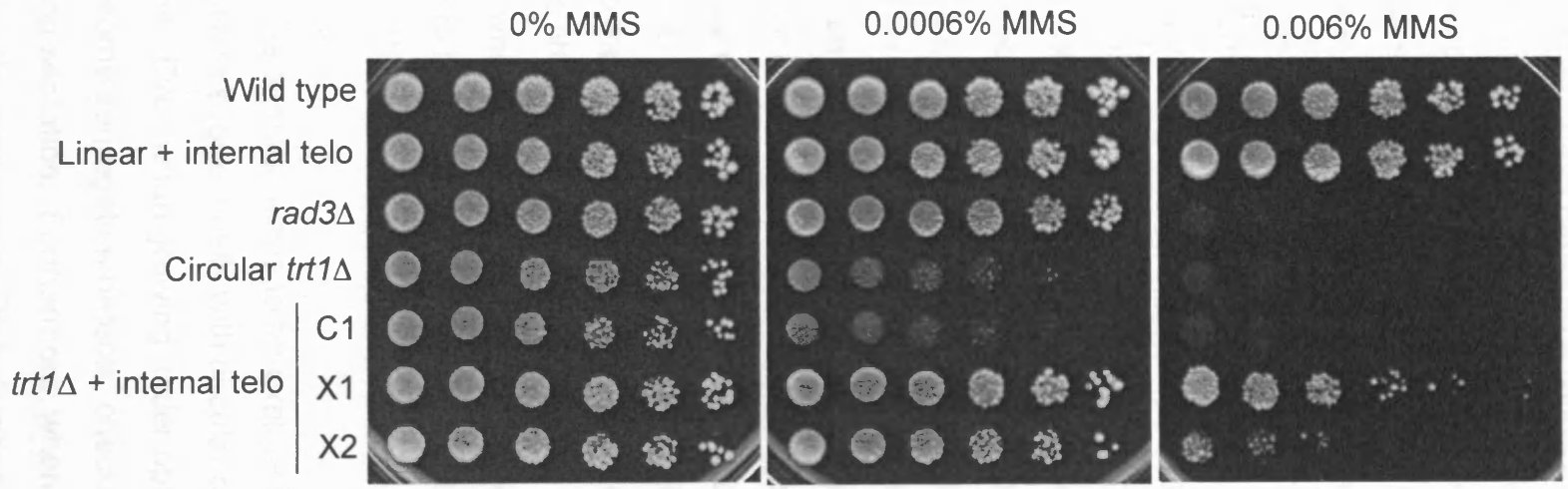


Figure 3.18 Three types of *trt1*Δ survivor with internal telomere with varying degrees of damage sensitivity

5-fold dilution assay of *trt1*Δ survivors with internal telomere sequence. 5μl of each dilution was spotted onto rich media with the indicated amounts of MMS. Survivors C1, X1 and X2 all contain internal telomere sequence but have different levels of damage sensitivity.

3.11 Conclusions

In this chapter I have explored a range of physiological situations that pose a problem for fission yeast strains that have survived loss of telomeric DNA through circularisation of each of the three chromosomes. Clearly, the lack of telomeres significantly compromises the cells in a range of situations, be it through the actual absence of telomere repeats themselves, lack of binding sites for telomere associated proteins, or the topologically unusual form the chromosomes are arranged in. Whether telomeres evolved to aid in these situations, or pathways required to cope with circular chromosomes have subsequently been lost during evolution due to redundancy is open for debate. Clearly the presence of circular chromosomes does not always pose such a problem; most prokaryotes are able to manage circular genomes. However, prokaryotes and eukaryotes have important differences that may account for ability of the former to cope with circular genomes. It would perhaps be naïve to think that the two situations could be directly comparable. One major difference is the size of the genome. It may be that the much larger size of the eukaryotic genomes are not sustainable as circular molecules. Another requirement of eukaryotic chromosomes is that they undergo meiosis. Meiosis with circular genomes is likely to lead to deleterious dicentric chromosomes following meiotic recombination. Alternatively, it may be that, while prokaryotes were able to cope with a linear genome, eukaryotes evolved to have a linear genome and did not acquire the machineries required to deal with a circular genome.

Each of the defects needs to be explored in greater detail. However, initial observations suggest cells with circular chromosomes display many problems. Even when growing under optimal conditions, circular strains show chromosome segregation defects, checkpoint activation and problems with regulating septation. Furthermore, when grown under conditions of stress, such as in the presence of DNA damage or during meiosis, circular strains display an extreme loss of viability.

While the presence of internally placed telomeres, either on a plasmid in high copy number, or as a single copy within the genome, does not aid survival following DNA damage, the linearisation of a single chromosome with the addition of telomere repeats partially suppresses the defect. Whether this is due to the presence of *bona fide* telomeres, the alleviation of a topological problem or a combination of the two cannot be distinguished at present. However, it is interesting to note that the overexpression of topoisomerases does not help circular strains cope with DNA damage.

Through investigating the damage sensitivity of circular strains, I have also uncovered two survivors in the absence of telomerase with greatly suppressed damage sensitivity. Through initial analysis, these strains have survived through circularisation of all three chromosomes. Understanding the suppression of damage sensitivity and the basis of survival of these strains is the focus of my next chapter.

4 Characterisation of two telomerase negative strains with a novel mode of survival

4.1 Introduction

Telomerase plays an important role, allowing the continued maintenance of telomeres and hence the continued proliferation of cells. A broadening range of survival mechanisms in the absence of telomerase is becoming apparent in different model systems as discussed in greater detail in Chapter 1. Most mechanisms involve the amplification of terminal telomere sequences by recombination-based mechanisms. In budding yeast, the major types of survivors observed in the absence of telomerase are known as type I and type II survivors. Type I maintain linear chromosomes through amplification of the subtelomeric Y' elements, retaining short telomere sequences at chromosome ends (Lundblad and Blackburn, 1993). Type II survivors maintain long, heterogeneous telomeres (Teng and Zakian, 1999). In immortalised human cell lines, cells maintain telomeres in the absence of telomerase by a recombination-based mechanism known as ALT (Alternative Lengthening of Telomeres) (Bryan et al., 1995; Dunham et al., 2000).

In fission yeast, however, survival without telomerase occurs primarily by a different mechanism. The small number of chromosomes allows survival following processing of chromosome ends as breaks. Each of the three chromosomes undergo intramolecular fusion forming individual circles, having lost all telomeric, and most of the subtelomeric DNA (Nakamura et al., 1998). In other organisms, the greater number of chromosomes means survival by this mechanism is impossible. Circular survivors are also seen in other fission yeast mutants defective for proteins involved in telomere maintenance, including *pot1*Δ and strains lacking both of the checkpoint kinases, Rad3 and Tel1 (Baumann and Cech, 2001; Naito et al., 1998). Survivors with linear chromosomes are also observed in fission yeast in the absence of telomerase, maintaining telomeres through recombination. In fact, this is the major mode of survival in liquid. Furthermore, this is the exclusive mode of survival following disruption of telomerase in cells lacking Taz1 (Nakamura et

al., 1998), indicating that Taz1 inhibits the formation of recombination-based survivors. Indeed the stalled replication forks seen at *taz1*Δ telomeres may stimulate survival by recombination (Miller et al., 2006). Circular *trt1*Δ*taz1*Δ strains can only be obtained by knocking out $trt1^+$ following chromosome circularisation of a *trt1*Δ survivor. Interestingly, in the presence of Taz1, recombination based survivors are unstable. Telomere shortening occurs and circular survivors eventually emerge (Nakamura et al., 1998).

In budding yeast lacking telomerase and recombination pathways, survival occurs through the formation of large palindromes at chromosome ends (Maringele and Lydall, 2004b). These cells survive maintaining linear chromosomes through forming large palindromic sequences at chromosome ends. Cells surviving by this so-called PAL mechanism are able to survive with reduced telomere signals, suggesting many of the highly rearranged chromosomes have lost the majority, if not all telomere DNA (Maringele and Lydall, 2004b).

In the previous chapter I described a general sensitivity of fission yeast strains with circular chromosomes to agents that induce damage. While investigating the role of telomeres in surviving damage, two survivors emerged in the absence of telomerase with greatly suppressed damage sensitivity. Through initial analysis, survival appeared to have occurred through the usual mechanism of chromosome circularisation. This chapter focuses on trying to understand the basis behind the suppressed sensitivity of these new survivors, X1 and X2. Further analysis demonstrated that, while these strains have many similarities to conventional circular survivors, there are also many clear differences. We suggest that X1 and X2 have survived by a novel mechanism, maintaining linear chromosomes in the absence of terminal telomere repeats. X1 and X2 have survived having amplified different types of heterochromatin; rDNA repeats in X1 and subtelomeric elements in X2. In trying to understand the survival of these strains, questions have also been thrown onto the circularity of chromosome III in conventional circular survivors. We propose that chromosome III does not undergo circularisation, but rather forms an alternate structure, maintaining a linear chromosome in

the absence of telomeres in a similar manner to all three chromosomes in X1 and X2.

4.2 X1 and X2 show an elongated phenotype and DAPI staining similar to circular strains

As described in Chapter 3, strains harbouring circular chromosomes show an elongated phenotype suggestive of checkpoint activation. In a similar manner, survivors X1 and X2 also have many elongated cells (Figure 4.1 A). As with circular strains, there are also many dead cells in a culture, the cells dying without elongation suggesting that the cell cycle delay is required for survival. DAPI staining suggests X1 and X2 have chromosome segregation defects in a similar manner to a sensitive circular strain (Figure 4.1 B). Cells are frequently observed with chromosome bridges and also acentric nuclei. There are also many cells in an exponentially growing culture that have multiple septa, as with circular strains, suggesting a problem with the septation initiation network (SIN) (Figure 4.1 A). Hence, by initial observation, these strains' morphology appears similar to that of a circular strain. It was notable, however, that the generation time for X1 and X2 was slightly improved compared with a damage sensitive circular strain, decreasing from 5.47 hours for C1 to 3.44 hours for X1 and 4.09 hours for X2 in rich media. This is still increased compared with a wild type strain, having a generation time of 2.11 hours.

4.3 X1 and X2 show a suppressed sensitivity to a range of damaging agents

As described in Chapter 3, strains with circular chromosomes show sensitivity to a range of damaging agents. However X1 and X2 emerged as apparently circular telomerase negative survivors with suppressed sensitivity to MMS. We looked at the sensitivity of these strains to the same range of damaging agents conventional circular strains are sensitive to, so we could further assess the similarities and differences to conventional circular strains. Looking at a similar range of damaging agents, we observed that the new *trt1*Δ survivors display a suppression in sensitivity similar to that observed for MMS. When grown on plates containing bleomycin or hydroxyurea, both X1

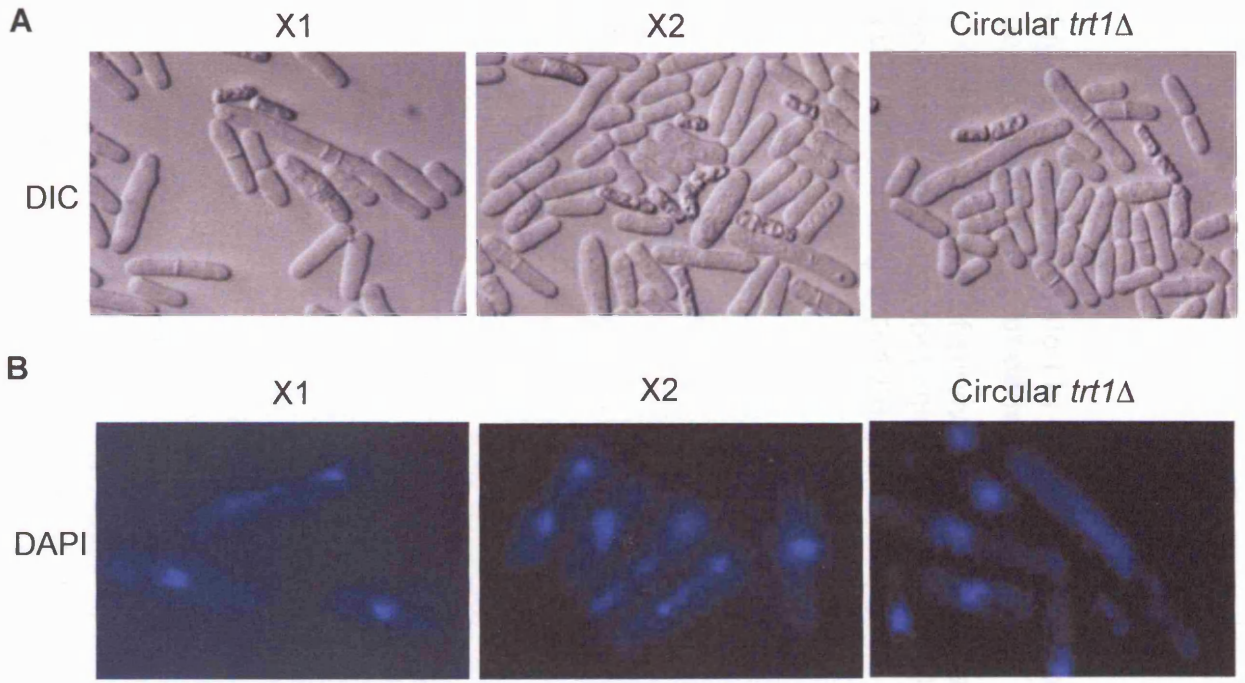
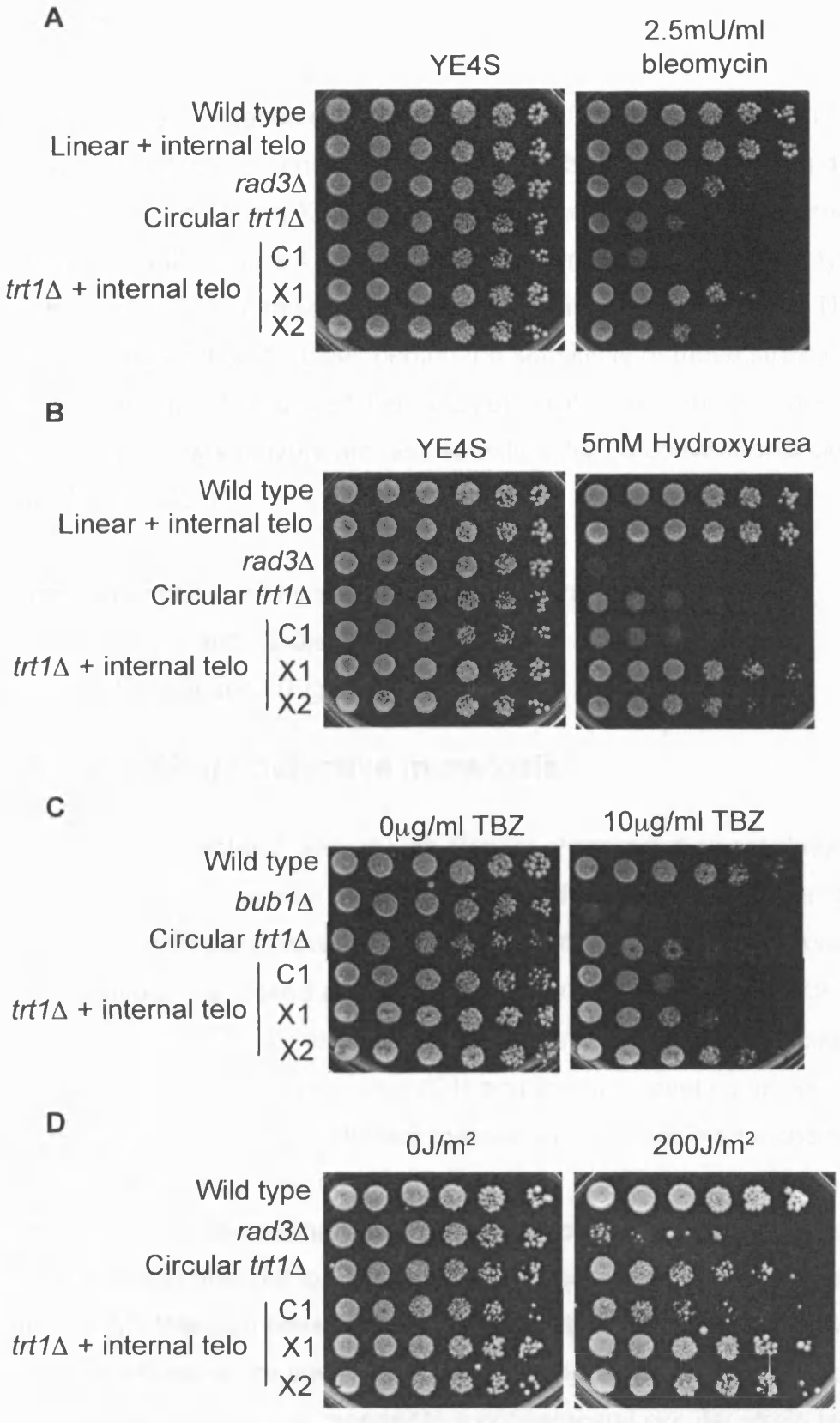


Figure 4.1 X1 and X2 show an elongated phenotype and DAPI patterns suggestive of chromosome segregation defects

(A) DIC pictures of X1, X2 and a damage sensitive circular *trt1*Δ strain. In a similar manner to a damage sensitive strain, X1 and X2 cultures also have many elongated, dead and multiseptated cells.
(B) DAPI staining shows X1 and X2 have chromosome segregation defects, as with a damage sensitive circular *trt1*Δ strain.

Figure 4.2 Suppression in drug sensitivity of X1 and X2 is general for a range of damaging agents

5-fold serial dilution of survivors with an internal telomere sequence on plates containing bleomycin (A), hydroxyurea (B), TBZ and following exposure to UV radiation.


A
YE4S
2.5mU/ml bleomycin
Wild type
Linear + internal telo
rad3Δ
Circular trt1Δ
trt1Δ + internal telo
C1
X1
X2
B
YE4S
5mM Hydroxyurea
Wild type
Linear + internal telo
rad3Δ
Circular trt1Δ
trt1Δ + internal telo
C1
X1
X2
C
0μg/ml TBZ
10μg/ml TBZ
Wild type
bub1Δ
Circular trt1Δ
trt1Δ + internal telo
C1
X1
X2
D
0J/m2
200J/m2
Wild type
rad3Δ
Circular trt1Δ
trt1Δ + internal telo
C1
X1
X2

and X2 displayed a sensitivity that was far less pronounced than that of a conventional circular strain. There was also a difference in sensitivity between X1 and X2; X2 had a slightly greater loss of viability than X1 (Figure 4.2 A and B).

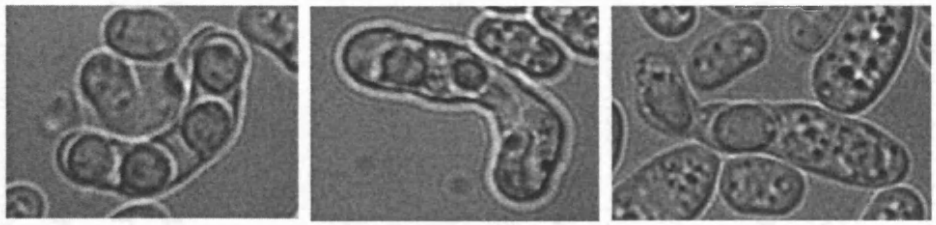
Because of the segregation defects observed by DAPI staining X1 and X2, one might expect to see sensitivity to the microtubule depolymerising drug, TBZ. Indeed, both X1 and X2 displayed a slight sensitivity to TBZ compared with wild type cells (Figure 4.2 C). Interestingly, in contrast the other types of damage where X2 is more sensitive than X1, X1 is more sensitive to TBZ than X2. This suggests that the basis behind the sensitivity of these strains to DNA breaks is different to that underlying TBZ sensitivity. As with the other types of damage, the new survivors are less sensitive than a conventional circular survivor (Figure 4.2 C).

In a similar manner to that we observed with a strain with circular chromosomes, X1 and X2 display only a minimal sensitivity following exposure to UV radiation (Figure 4.2 D).

4.4 X1 and X2 are defective in meiosis

As described in Chapter 3, strains with circular chromosomes are defective in meiosis. To further ascertain the differences and similarities between the *trt1*Δ survivors with suppressed damage sensitivity and the conventional circular survivors, we looked at the meiotic ability of strains X1 and X2. In a similar manner to a circular strain lacking telomere repeats, both circular strains with a single telomere repeat (C1) and the two novel survivors (X1 and X2) were unable to complete efficient meiosis when mated with a linear wild type strain (Figure 4.3). For all types of survivor, meiosis proceeded to ascus formation as with conventional circular strains, however many of the asci were aberrant, showing unusual looking spores and spore numbers other than four (Figure 4.3 A). We also observed the spores that do form are often nonviable. Figure 4.3 B shows spore viability following plating an estimated 300 spores onto rich media. In a similar manner to a conventional circular strain lacking telomere sequences, a cross between C1 and a linear strain shows a drop in spore viability to about 13%. Strain X2 displayed a similar loss of spore

A



Wild type x wild type | X1 x wild type | X2 x wild type

B

wild type linear x wild type linear	Circular *trt1*Δ x wild type linear	C1 x wild type linear	X1 x wild type linear	X2 x wild type linear
97.3	15	13.3	40.3	18

Figure 4.3 Meiosis in survivors X1 and X2 is defective

(A) Examples of aberrant asci from meiosis involving X1 and X2.

(B) Spore viability following meiosis of linear strains with each of the *trt1*Δ survivors. 300 spores were plated on rich media and colonies counted. Numbers are recorded as the percentage colonies formed compared with the number of sores plated.

viability, showing about 18% colony formation. An increase in spore viability to about 40% compared with conventional circular strains was observed following meiosis between X1 and a wild type linear strain. However this is still greatly reduced compared with meiosis between two wild type strains. The spore viabilities described here for the strains with defective meiosis are likely to be an underestimate. The aberrant spores formed are often hard to distinguish from the cell debris so only those that were obviously spores were counted. Therefore it is likely that more than 300 spores may have been plated, bringing the viability figures down.

4.5 Survivors X1 and X2 display PFGE patterns different to conventional circular survivors

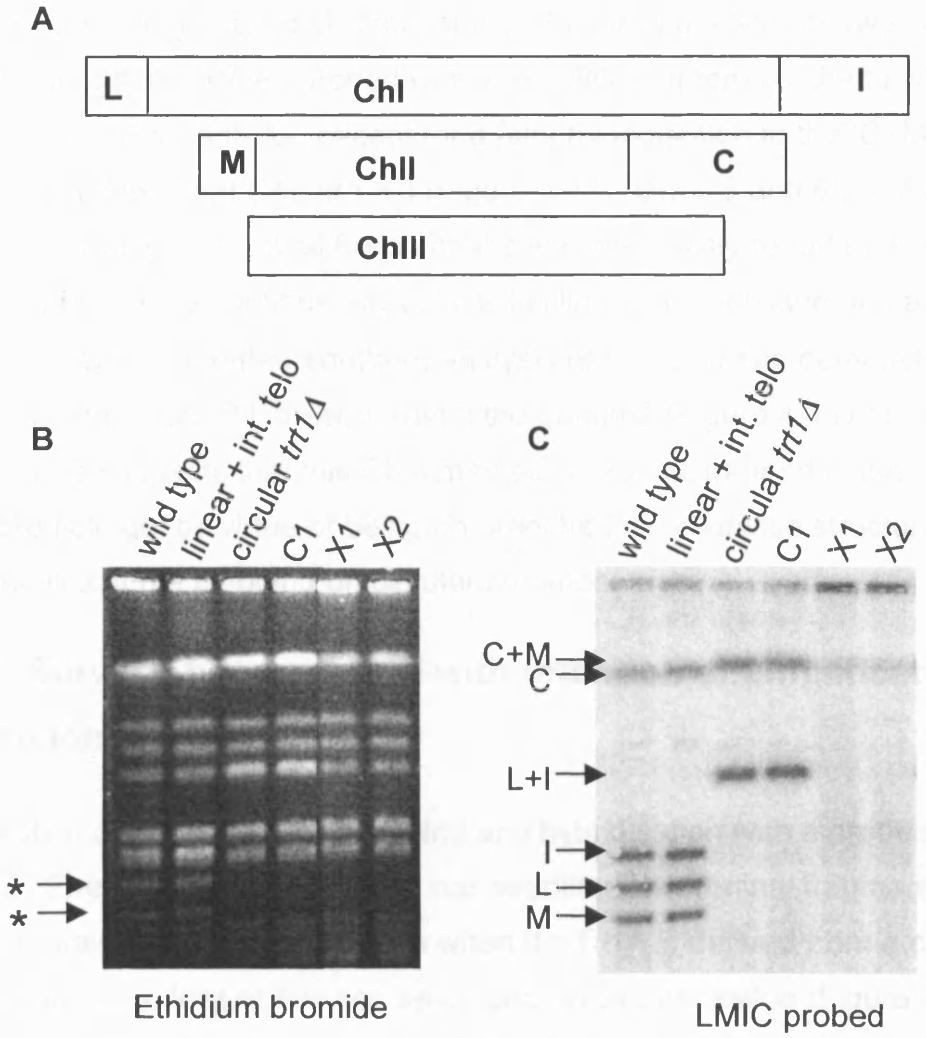
Pulsed field gel electrophoresis (PFGE) is routinely used to identify circular chromosomes. PFGE of whole chromosomes resolves the three linear chromosomes. However, circular chromosomes are unable to enter a pulsed field gel; the DNA remains trapped in the well. As described in Chapter 3, whole chromosome pulsed field gel analysis of X1 and X2 shows no entry of any of the three chromosomes, initially suggesting the cells have survived loss of telomerase through circularisation of all three chromosomes (Figure 3.17 A).

Another way circularisation is detected is by assessing the appearance of fusion fragments. To accomplish this we digest genomic DNA with NotI, a rare cutting enzyme. Digestion in agarose plugs for PFGE analysis liberates the terminal, telomere containing fragments of chromosomes I and II with a definable size (Figure 4.4 A). Chromosome III remains uncut. Looking at the ethidium bromide stained gel, we can see that bands known to be terminal fragments are absent in strains with circular chromosomes, running as higher molecular weight fusion bands (Figure 4.4 B, bands marked with an *). Similarly, in survivors X1 and X2, these bands are absent, again suggesting chromosome circularisation (Figure 4.4 B).

Southern analysis of the NotI pulsed field gel using probes specific to the L, M, I and C fragments shows the four terminal fragments as discrete bands in a linear strain (Figure 4.4 C, lanes 1 and 2). Similar NotI analysis of a strain

Figure 4.4 X1 and X2 display PFGE patterns different to conventional circular survivors

(A) Schematic representation of terminal NotI restriction sites in chromosomes of *S. pombe*.
(B) Ethidium bromide stained NotI pulsed field gel shows X1 and X2 have lost terminal fragments (marked *) as with a conventional circular strain.
(C) Southern analysis of terminal NotI restriction fragments shows hybridisation patterns different to conventional circular survivors. Terminal fragment probes do not hybridise in X1 and X2, except faintly at the band representing C+L/M/I.
(D) PCR analysis demonstrates that these terminal regions are in fact still present within the gel, but not entering the gel.


A
L
ChI
I
M
ChII
C
ChIII
B
wild type
linear + int. telo
circular trt1Δ
C1
X1
X2
*
*
Ethidium bromide
C
wild type
linear + int. telo
circular trt1Δ
C1
X1
X2
C+M
C
L+I
I
L
M
LMIC probed
D
wt
Circular trt1Δ
X1
X2
L M I C L M I C L M I C L M I C

containing circular chromosomes allows the DNA to enter the gel. Rather than four bands, two bands running as the fusions of 'L+I' and 'C+M' are observed (Figure 4.4 C, lanes 3 and 4). Despite the ethidium bromide staining pattern of NotI digested DNA of X1 and X2 being reminiscent of a strain containing circular chromosomes, Southern analysis showed a very different pattern. When probed with the 'LMIC' sequences, the fusion bands are absent in X1 and X2, except for a faint hybridisation to the 'C+M' band (also may represent C+L or C+I) (Figure 4.4 C, lanes 5 and 6). A similar disappearance of terminal fusion bands was previously reported when circular *trt1*Δ strains were continuously cultured in liquid media (Baumann and Cech, 2000). PCR and further southern analysis of these strains demonstrate these regions are in fact still present within the genome (Figure 4.4 D and appendix A2). This suggests that this DNA may somehow be trapped in the well of the pulsed field gel by virtue of being on branched or otherwise structured DNA molecules, if not by being on circular chromosomes.

4.6 Survival has occurred with retention or amplification of subtelomeric elements

Digestion of genomic DNA with NotI and hybridisation with a probe specific to the STE region yields four bands representing the terminal fragments in a linear strain, and no hybridisation when the DNA is derived from a circular survivor due to loss of this sequence prior to circularisaiton (Figure 4.5). We also see a faint signal in the well of the internal telomere survivors. This is due to the 32bp STE sequence integrated with the telomere sequence at the *ura4* locus of chromosome III which contains no NotI sites. This pattern was also observed with survivor X1, suggesting loss of STE during survival (Figure 4.5). However, survivor X2 displayed a very unexpected pattern; the STE sequences were present throughout the genome, hybridising to each of the NotI ethidium bands (Figure 4.5). This suggests that during survival, telomere associated sequences have spread throughout the genome, at least through chromosomes I and II.

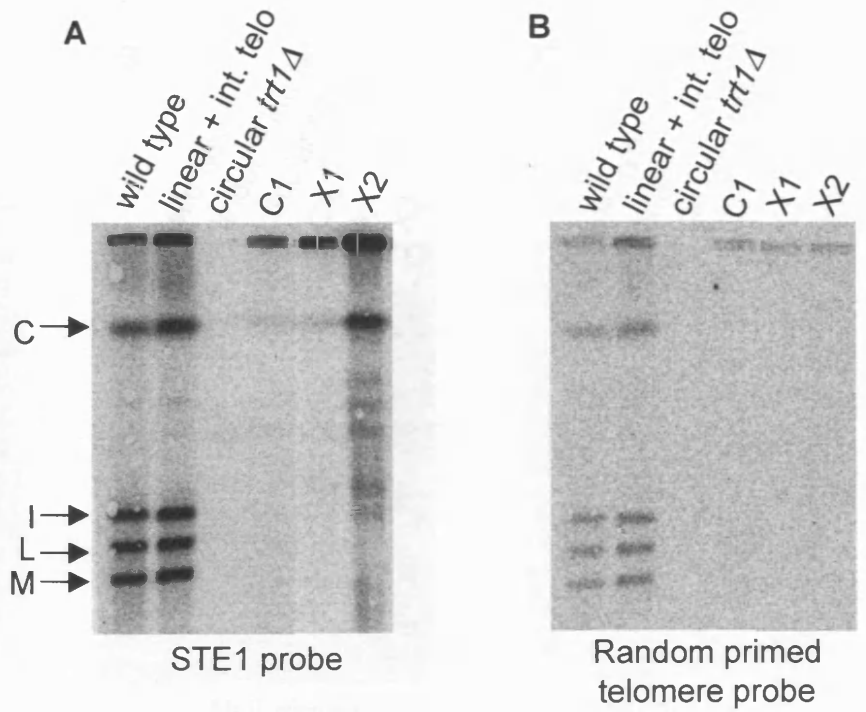


Figure 4.5 Southern analysis of Not1 digested chromosomes

(A) Hybridisation of NotI Southern with a STE1 probe demonstrates X2 has undergone significant amplification of this region throughout the genome.
(C) Hybridisation with a telomere specific probe suggests these strains have not retained telomere DNA. Faint hybridisation within the well represents the internal telomere integrated in chromosome III at *ura4*.
For Ethidium gel, see figure 4.4 B

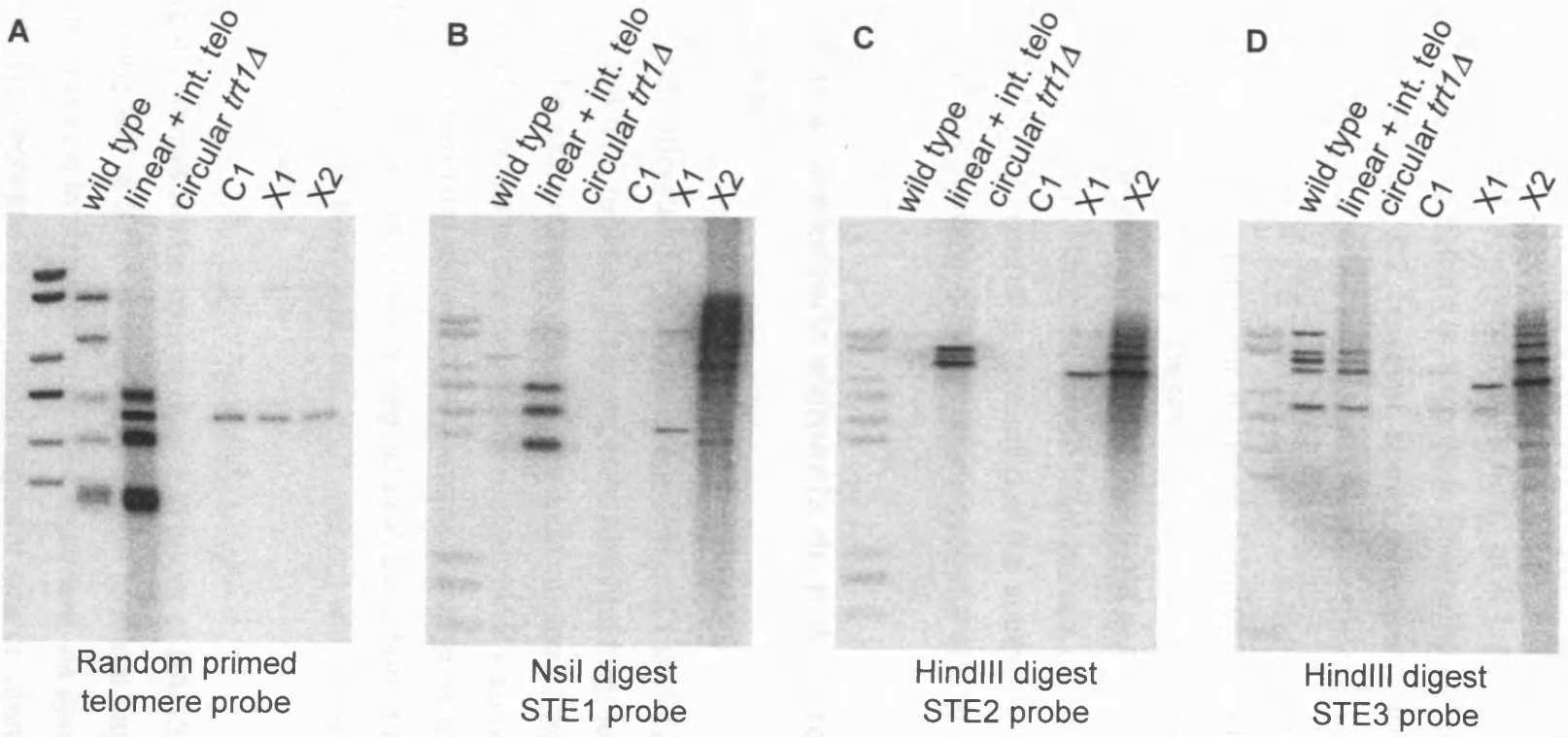


Figure 4.6 X1 and X2 have retained or amplified different amounts of subtelomeric DNA

Southern analysis demonstrates X1 has retained subtelomeric elements and X2 has amplified subtelomeric elements, but not telomere sequence. Southerns of restriction enzyme digested genomic DNA using the indicated restriction digests, probing with telomere (A), STE1 (B), STE2 (C) and STE3 (D).

Further analysis of the survivors shows the two strains have undergone varying degrees of STE amplification and/or retention during survival. NsiI digest of wild type genomic DNA liberates the terminal fragment containing telomere and STE1 (Figure 3.11). In a wild type strain, four discrete telomere specific bands are observed (Figure 4.6). In X.1, two STE1 specific bands are observed, and the pattern is mirrored in Southerns specific for STE2 and STE3 (Figure 4.6). X.2, however, shows a huge amplification of STE1 DNA, as also reflected with the NotI pulsed field gel Southern (Figure 4.5). This huge amplification of STE sequences in X.2 is extended into STE2 and STE3 regions (Figure 4.6). Interestingly, upon disruption of telomerase in budding yeast, activation of the Ty1 transposable element is observed (Scholes et al., 2003). The ty1 element has been shown to be involved in the mobilization of the subtelomeric Y' elements in type I telomerase deficient survivors (Maxwell et al., 2004). The extensive spreading of the subtelomeric sequences throughout the genome of X2 is reminiscent of a similar retrotransposon-type amplification.

4.6.1 The subtelomeric elements do not represent terminal fragments

To help us understand the mode of survival of X1 and X2, we wanted to know if any of the STE regions observed were terminal fragments. To look at this we used the exonuclease, BAL-31. BAL-31 nuclease digests DNA from termini, allowing us to see degradation of terminal fragments. Reactions included a linearised plasmid DNA control, allowing us to monitor the action of the nuclease and the extent of degradation occurring at each time point. Following BAL-31 treatment, the DNA was cut with a restriction enzyme for Southern analysis.

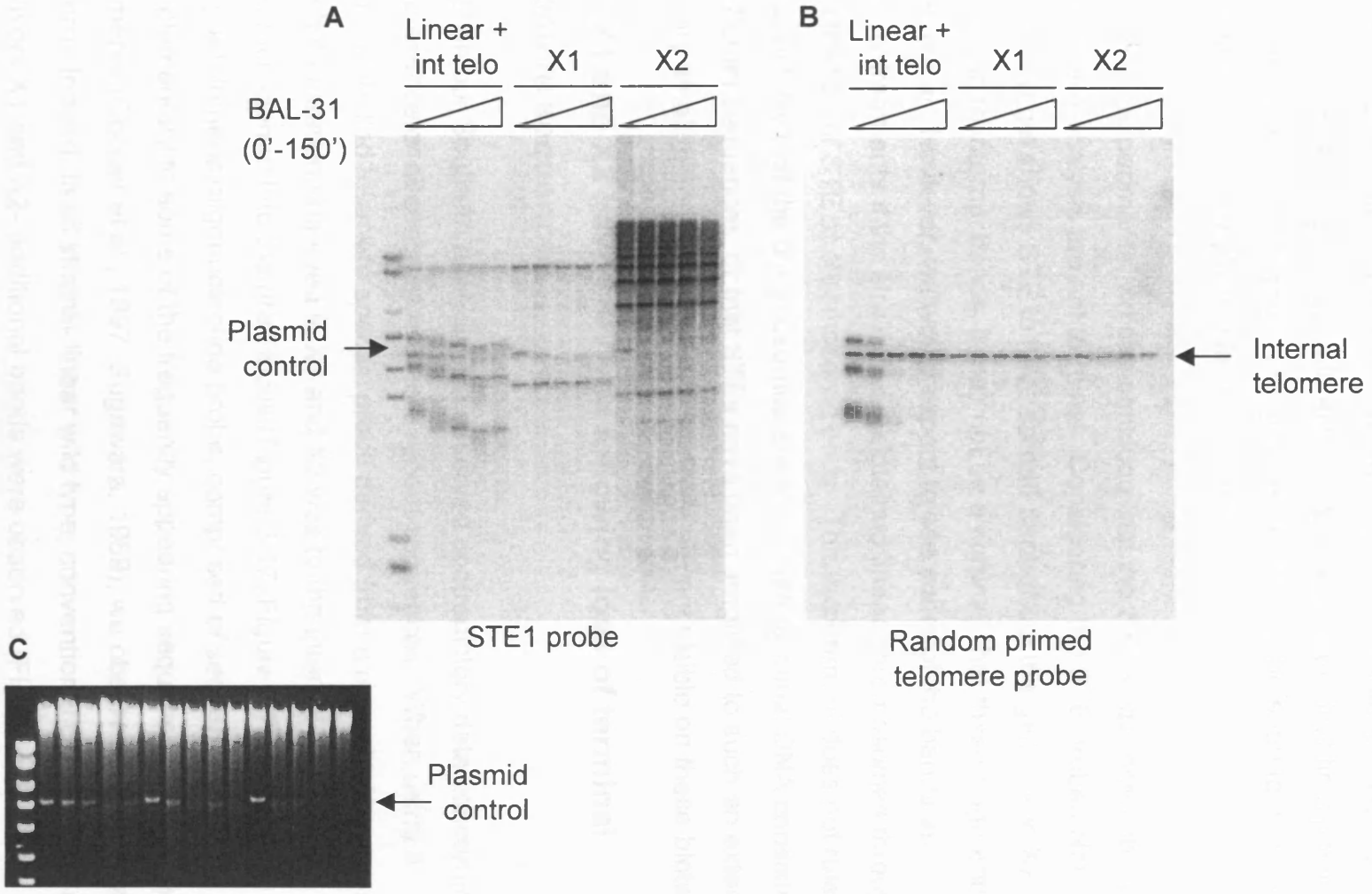
Figure 4.7 shows a time course over 2 ½ hours of BAL-31 treatment. Hybridising with a STE1 probe, the wild type terminal fragments can clearly be seen decreasing in size with increased treatment, as can the linear plasmid control. No decrease in restriction fragment size or intensity can be observed for any of the bands in strain X1 or X2 (Figure 4.7 A). A telomere-specific probe was used to look at the internal telomere as a control for loading and to ensure there was no endonuclease digestion occurring upon treatment

Figure 4.7 STE fragments are not terminal

(A) Digestion with BAL-31 nuclease over a 2.5 hour time course, followed by NsiI digestion and Southern analysis using a STE1 specific probe demonstrate the subtelomeric fragments are not terminal. Linearised plasmid control DNA can be seen to decrease in size and intensity with increased BAL-31 treatment as the DNA is digested, as can terminal fragments in the strain with linear chromosomes.

(B) Hybridisation of the same blot with a telomere specific probe shows equal loading using the internal telomere fragment as a standard.

(C) Ethidium bromide stained gel of BAL-31 digested DNA prior to NsiI digestion. Linearised plasmid control can be seen to decrease in size and intensity with increased BAL-31 treatment.


A
Linear +
int telo
X1
X2
BAL-31
(0'-150')
Plasmid
control
STE1 probe
B
Linear +
int telo
X1
X2
Internal
telomere
Random primed
telomere probe
C
Plasmid
control

(Figure 4.7 B). As many of the STE1 fragments have a high molecular weight, particularly in X2, a second BAL-31 experiment was carried out over a 7 hour period to allow greater digestion of any terminal fragments, and the gel run further for better resolution of larger fragments. In this case, the wild type terminal fragments are almost completely digested and a linear plasmid with a higher molecular weight is completely degraded within the first time point. However, none of the fragments in either X1 or X2 can be seen to decrease in either size or intensity (Appendix, Figure A3).

From these experiments, we can conclude that the STE sequences observed by Southern analysis are not terminal. Considering the STE probed NotI pulsed field gel shows STE to have spread throughout the genome in X2, hybridising to internal bands, it may not be a surprise that these fragments are not terminal. However, we would expect to see some of the bands as terminal fragments if the strain had maintained linear chromosomes through amplification of STE at chromosome ends. This experiment does not rule out the possibility that the chromosomes are linear with terminal DNA consisting of different sequences, or that STEs have been amplified to such an extent the very small minority in terminal fragments are not visible on these blots.

4.7 X1 and X2 have survived following loss of terminal telomere sequences

Through our Southern analysis, we observed contradictory data concerning the presence or absence of telomere repeat sequences. When using a random labelled telomere specific probe derived from a cloned telomere, the only hybridisation observed in X1 and X2 was to the internal telomere sequence cloned into the *ura4* locus (Figure 3.17, Figure 4.7). However, using a telomeric oligonucleotide probe, comprised of sequences complementary to some of the frequently appearing sequence in *S. pombe* telomeres (Cooper et al., 1997; Sugawara, 1989), we observed a different pattern. Indeed, in all strains- linear wild type, conventional circular *trt1*Δ and survivors X1 and X2- additional bands were observed (Figure 4.8). The additional bands were particularly striking in X2 (Figure 4.8). Because we use a lower temperature for hybridisation when using end labelled oligonucleotide probes compared with random labelled probes, we wondered if the difference

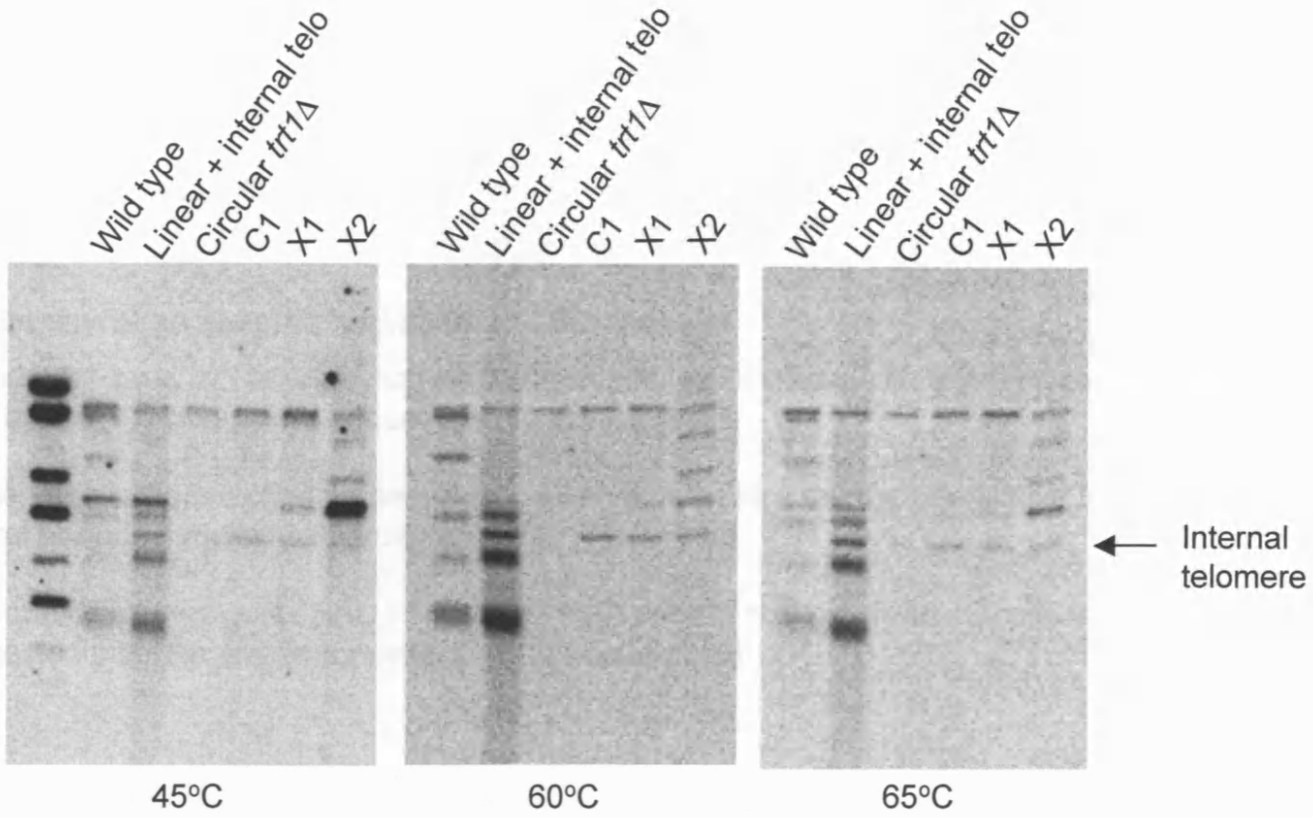


Figure 4.8 Temperature gradient of telomere oligo hybridisation.

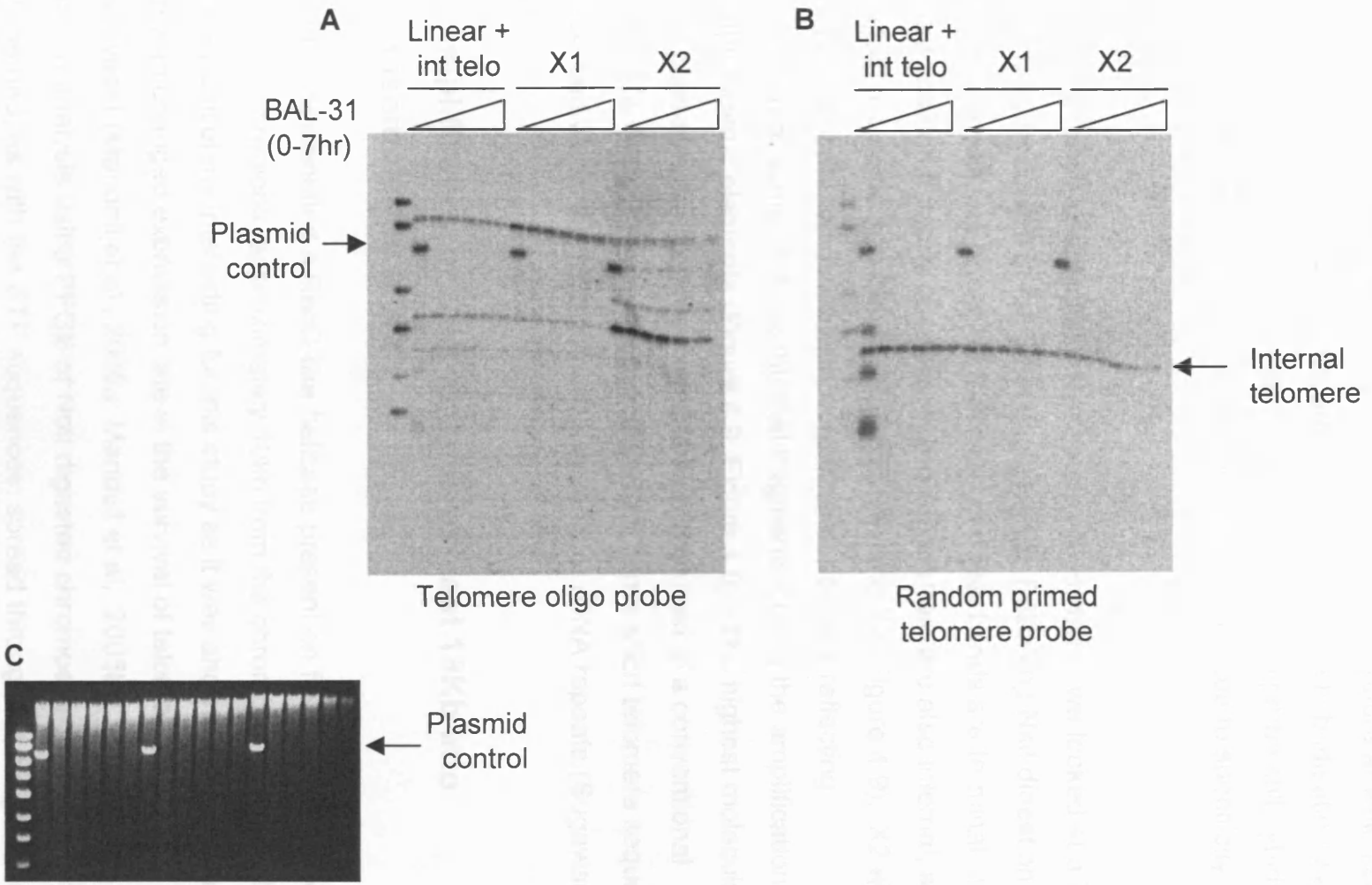
Hybridisation of telomere oligo probes to additional fragments compared with random labelled telomere oligo was observed. This pattern was not dependent on hybridisation temperature.

Figure 4.9 Telomere oligo specific fragments are not terminal

BAL-31 treatment followed by NsiI digestion and hybridisation of Southern with telomere oligo probe demonstrates telomere oligo specific sequences are not terminal.

(A) Hybridisation with telomere oligo probe shows no decrease in size or intensity relative to the internal telomere control (random labelled telomere probe, B).

(C) Ethidium bromide stained gel of BAL-31 digested DNA prior to NsiI digestion. Linearised plasmid is completely digested after the first time point following treatment with BAL-31.


A
Linear +
int telo
X1
X2
BAL-31
(0-7hr)
Plasmid
control
Telomere oligo probe
B
Linear +
int telo
X1
X2
Internal
telomere
Random primed
telomere probe
C
Plasmid
control

in hybridisation pattern observed was reflecting this, demonstrating a difference in stringency at the lower temperature. We carried out a hybridisation gradient between 45°C (the temperature normally used for telomere oligo hybridisation) and 65°C (the temperature used for random labelled probe hybridisation) to see if there was a temperature at which signal was lost for any of the bands, indicating that perhaps the hybridisation was non-specific. However, even at 65°C, the telomere oligo probe still hybidised to the additional bands suggesting the difference is not due to specificity (Figure 4.8).

To see if the 'oligo-specific' telomere bands are terminal, we looked at a 7 hour BAL-31 treatment of the DNA (Figure 4.9). Following NsiI digestion and Southern analysis, we observed that none of these bands are terminal. In fact, the additional bands observed in the linear strain are also internal, and are in fact the same size bands retained in X1 and X2 (Figure 4.9). X2 also displays additional bands that are not terminal, perhaps reflecting amplification of some of these internal fragments during the amplification of the subtelomeric elements (Figure 4.9, Figure 4.6). The highest molecular weight 'oligo-specific' telomere band is also observed in a conventional circular *trt1*Δ strain. These bands may reflect some short telomere sequences interspersed within the subtelomeric elements or rDNA repeats (Sugawara, 1989).

4.8 Amplification of DNA extends at least 18Kb into chromosome arms of X2

Recent work identified a RecQ-like helicase present on four out of the six subtelomeric regions, approximately 10kb from the chromosome end. The gene is particularly interesting for this study as it was shown to be the only gene with changed expression late in the survival of telomerase mutants in fission yeast (Mandell et al., 2005a; Mandell et al., 2005b). Interestingly, Southern analysis using PFGE of NotI digested chromosomes showed the helicase had, as with the STE sequences, spread throughout the genome (Figure 4.10). Therefore, at some point late in survival following disruption of *trt1*$^{+}$, at least 18 Kb (10kb STE + 8Kb RecQ-like gene) of the chromosome end was amplified many times and inserted within the rest of the genome.

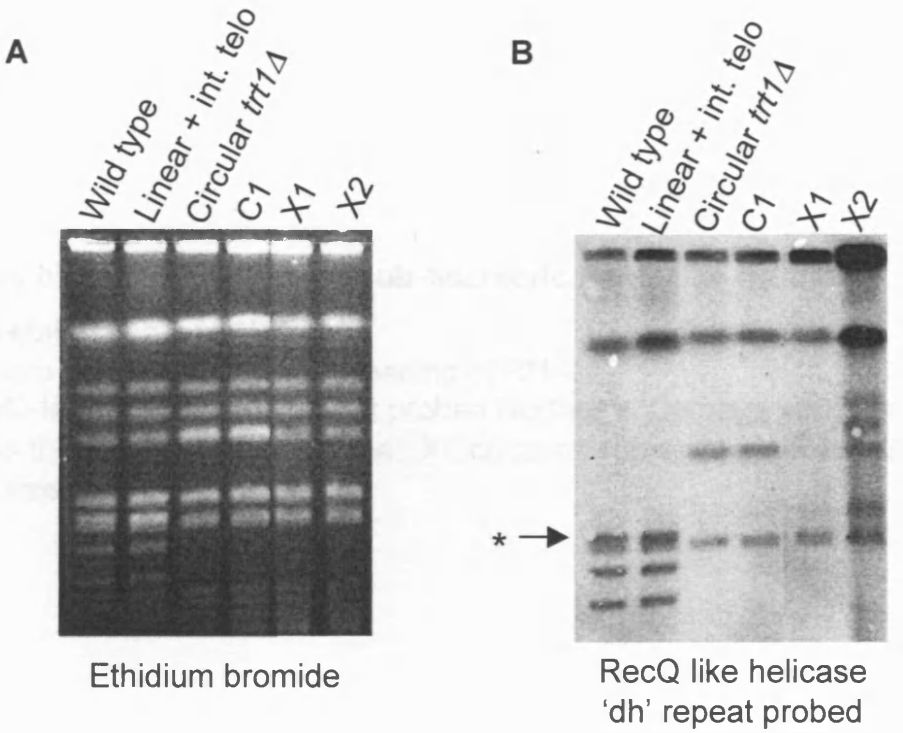
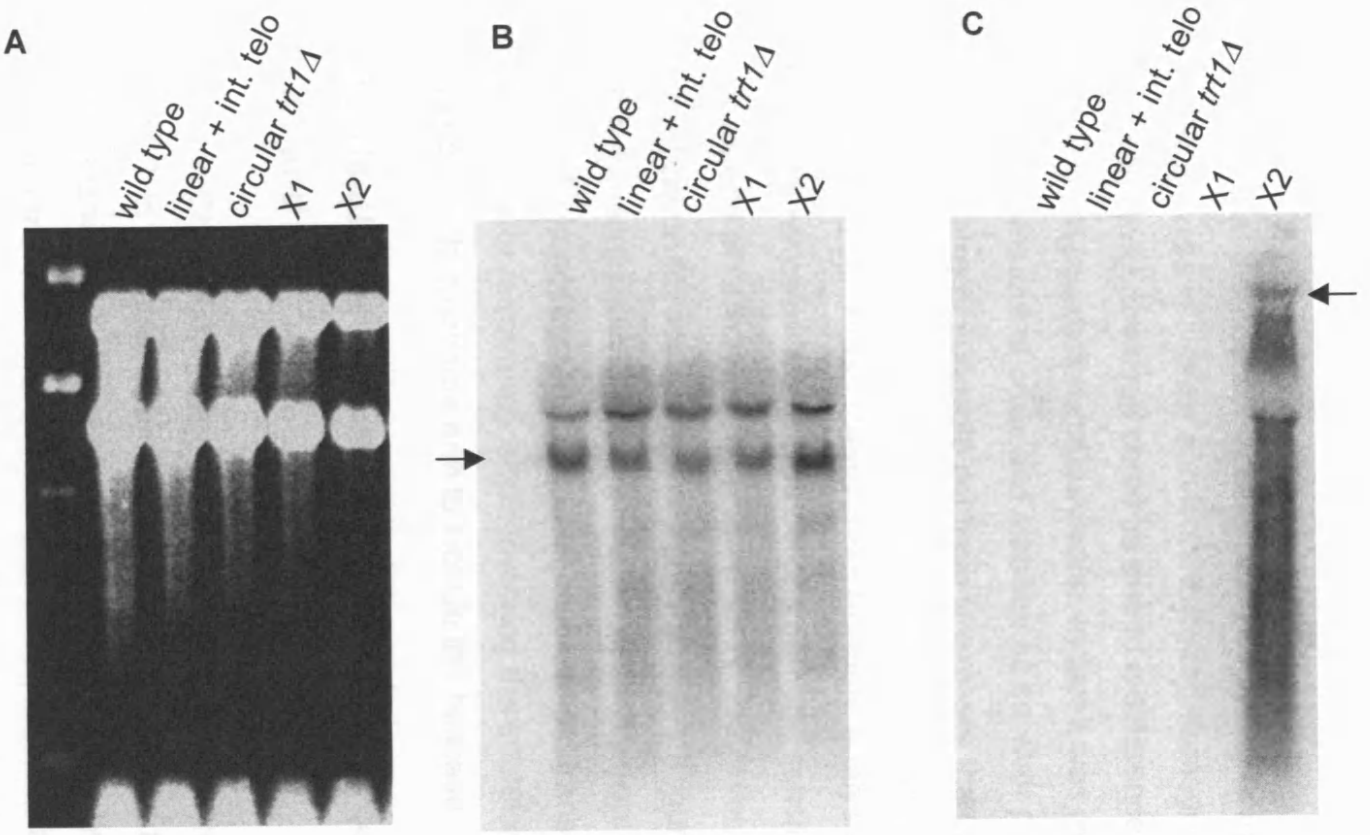


Figure 4.10 Amplification of DNA extends at least 18kb into chromosome arms of X2

(A) Ethidium bromide stained gel of NotI digested genomic DNA shows equal loading.
(B) Subtelomeric RecQ like helecase 'dh' repeat probed Southern demonstrates subtelomeric DNA amplification in X2 extends at least 18kb into chromosome arms. X1 also shows hybridisation at the C+L/M/I band size. * indicates hybridisation with the dh repeats in the centromeric region of chromosome I.

Figure 4.11 X2 shows high expression of a sub-telomeric RecQ-like helicase.

(A) Ethidium bromide stained gel of RNA.
(B) act1 probed Northern demonstrates equal loading of RNA.
(C) Subtelomeric RecQ-like helicase 'dh' repeat probed Northern. Damage sensitive circular *trt1*Δ survivor and X1 do not express the subtelomeric helicase. X2 contains subtelomeric RecQ-like helicase RNA transcripts of varying size.



Ethidium bromide

act1 probed

RecQ like helicase
'dh' repeat probed

We cannot be sure, however, if this amplification is involved in the survival mechanism and supression of damage sensitivity, or coincidental. We also observed strain X1 hybridised to the RecQ like helicase dh repeat probe at the C+L/M/I size, as is mirrored with the 'LMIC' probe (Figure 4.10, Figure 4.4 B).

It is conceivable that the helicase activity could be aiding or indeed interfering with the repair process in circular survivors following damage, and the difference in sensitivity observed could be due to a difference in expression of the gene in different populations of survivors. In fact it was reported that as the *trt1*Δ strains come out of crisis and stabilise as survivor populations, only a sub-population of circular survivors continue to express the helicase (Mandell et al., 2005a). We therefore decided to look at the expression of the helicase in the different *trt1*Δ survivors. Carrying out Northern analysis, we did not see expression of the helicase in wild type cells, a damage sensitive circular *trt1*Δ survivor, or indeed *trt1Δ* survivor X1 (Figure 4.11). However, X2 showed intense hybridisation across a range of transcript sizes (Figure 4.11). Whether this transcript is involved in the survival mechanism or is simply a consequence of it is not known. The RNA could reflect transcripts from an intermediate stage in retrotransposition involving the subtelomeric DNA and extending into the chromosome arm to include the helicase.

4.9 Linearisation of chromosomes upon reintroduction of *trt1*$^+$ to X1 and X2

As described in Chapter 3.7, reintroduction of *trt1*$^+$ causes linearisation of chromosome III in a circular strain. Following expression of telomerase in X1 and X2, the cells were no longer elongated and had a wild type morphology. Interestingly, in both X1 and X2, expression of *trt1*$^+$ allowed all three chromosomes to enter a pulsed field gel (Figure 4.12 A). In these cases, we observed extensive rearrangements of all three chromosomes. Probing with chromosome specific probes, we identified each of the three chromosomes entering the gel (Figure 4.12). A telomere specific probe confirmed 'linearisation' of each chromosome through addition of telomere repeats (Figure 4.12 B). We were also able to verify that in X2 the telomere associated sequences were present and greatly amplified on each

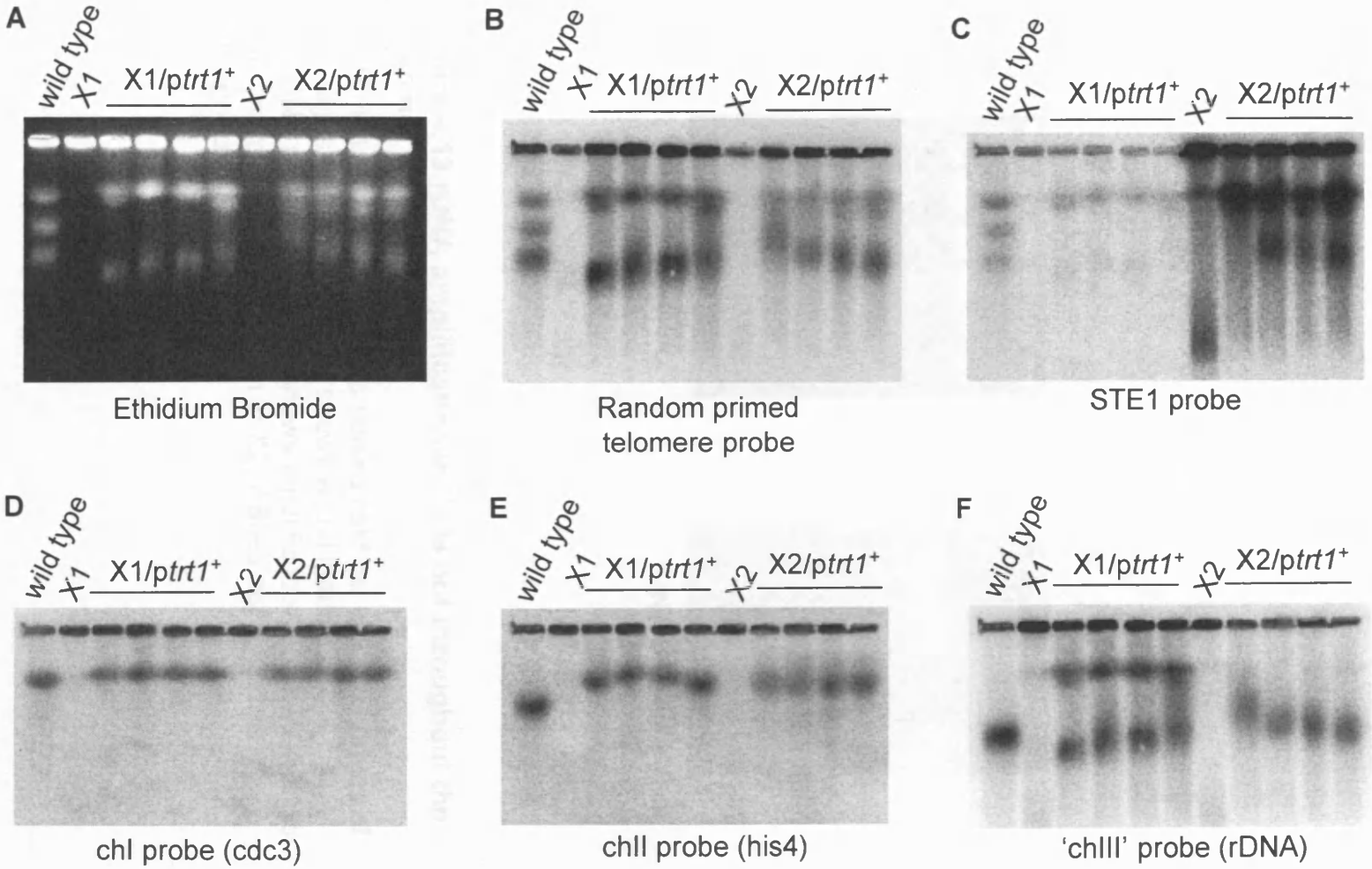
Figure 4.12 Reintroduction of telomerase to X1 and X2 causes linearisation of all three chromosomes

(A) Ethidium bromide stained gel of whole chromosome pulsed field gel electrophoresis. Reintroduction of *trt1*$^{+}$ to X1 and X2 allows entry of chromosomes into a pulsed field gel. Chromosomes have undergone extensive rearrangements as shown by the variation in size of band.

(B)Telomere probed Southern of gel in (A) demonstrates chromosomes have linearised with the addition of telomere repeats.

(C)STE1 probed Southern shows retention of subtelomeric elements in chromosomes of X1 and huge amplification of subtelomeric elements throughout each chromosome in X2.

(D)– (F) Hybridisation with chromosome specific probes shows each of the three chromosomes enter the gel and have undergone extensive rearrangements. 'Chromosome III specific' rDNA probe shows X1 has undergone rearrangements of the rDNA repeats to each of the three chromosomes.


A
wild type
X1
X1/p*trt1*$^+$
X2
X2/p*trt1*$^+$
Ethidium Bromide
B
wild type
X1
X1/p*trt1*$^+$
X2
X2/p*trt1*$^+$
Random primed
telomere probe
C
wild type
X1
X1/p*trt1*$^+$
X2
X2/p*trt1*$^+$
STE1 probe
D
wild type
X1
X1/p*trt1*$^+$
X2
X2/p*trt1*$^+$
chI probe (cdc3)
E
wild type
X1
X1/p*trt1*$^+$
X2
X2/p*trt1*$^+$
chII probe (his4)
F
wild type
X1
X1/p*trt1*$^+$
X2
X2/p*trt1*$^+$
'chIII' probe (rDNA)

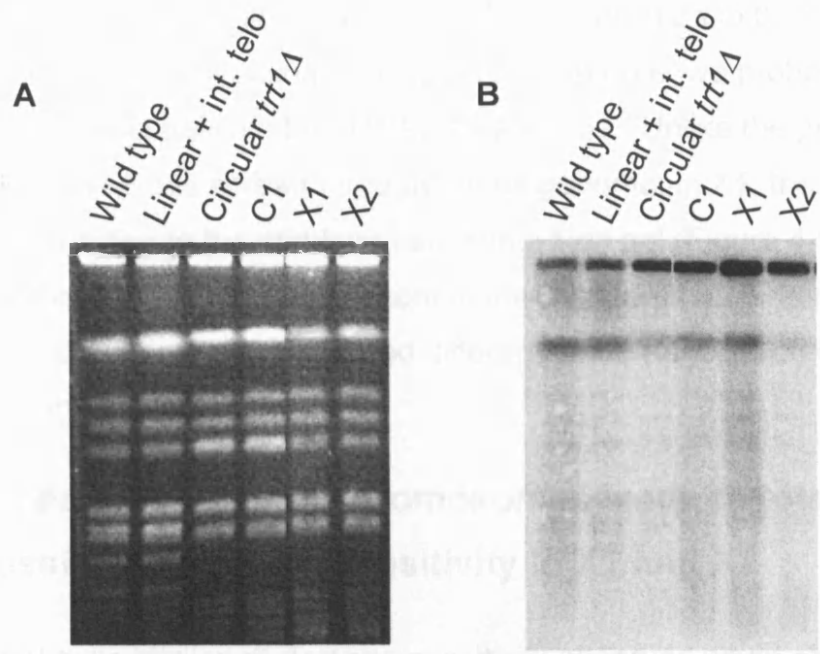


Figure 4.13 rDNA amplification in X1 is not throughout the genome

(A) Ethidium bromide stained pulsed field gel electrophoresis of NotI digested genomic DNA shows equal loading.
(B) rDNA probed Southern shows amplification of rDNA in X1 is different to STE amplification in X2; it does not hybridise throughout the genome.

chromosome (Figure 4.12 C). Surprisingly, it became apparent that X1 has undergone rearrangements of the rDNA repeats so that they are present on all three chromosomes rather than just chromosome III (Figure 4.12 F). We found this pattern very intriguing; we were faced with a scenario whereby survivor X1 had survived apparently amplifying one type of heterochromatic DNA (rDNA repeats) and survivor X2 amplifying another type (STE). To see if X1 also displayed a similar pattern of rDNA amplification to the STE amplification in X2, spreading throughout the genome, we probed the NotI digest pulsed field gel with the rDNA repeat probe. Unlike the pattern seen in X2 whereby STE has spread throughout the genome, in X1, the rDNA probe does not hybridise to the ethidium bands in a NotI gel (Figure 4.13). This suggests the repeats are only present in the fragments not entering the gel and the amplification is by a method different to the transposon-like amplification seen in X2.

4.10 Linearisation of all chromosomes leads to total suppression of damage sensitivity in X1 and X2

The partial suppression of damage sensitivity observed when chromosome III is linearised in a 'normal' circular survivor lead us to ask if linearisation of all three chromosomes with the addition of telomeres in X1 and X2 would be sufficient to suppress the low level damage sensitivity observed in these new survivors. This would help us understand if the sensitivity was due to the lack of telomere repeats and alternate chromosome topology, or due to the rearrangements during survival. A dilution assay on MMS showed that restoring the telomeres to X1 and X2 suppresses the damage sensitivity to a wild type level (Figure 4.14). This indicates that the sensitivity of these strains is due to lack of telomeres or the consequential alternate chromosome topology rather than caused by the rearrangements these strains have undergone.

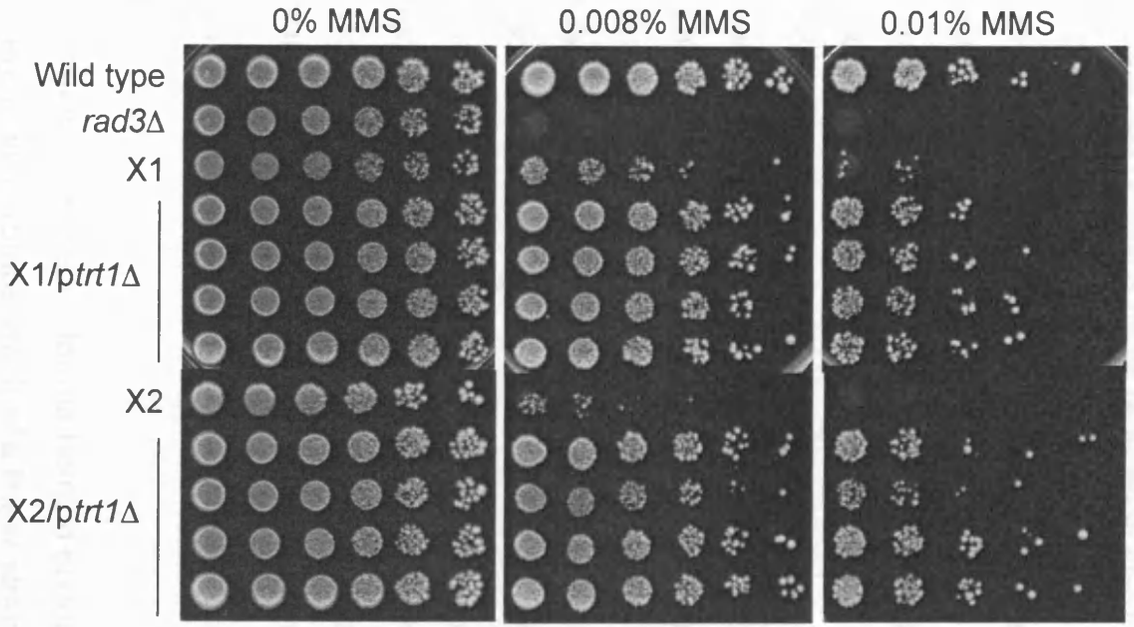


Figure 4.14 Reintroduction of telomerase to X1 and X2 completely suppresses the damage sensitivity

5-fold serial dilution assay on plates containing MMS. Addition of telomere repeats to each chromosomes suppresses the damage sensitivity of X1 and X2 to wild type level.

4.11 Mechanically opening chromosomes does not allow entry into a pulsed field gel

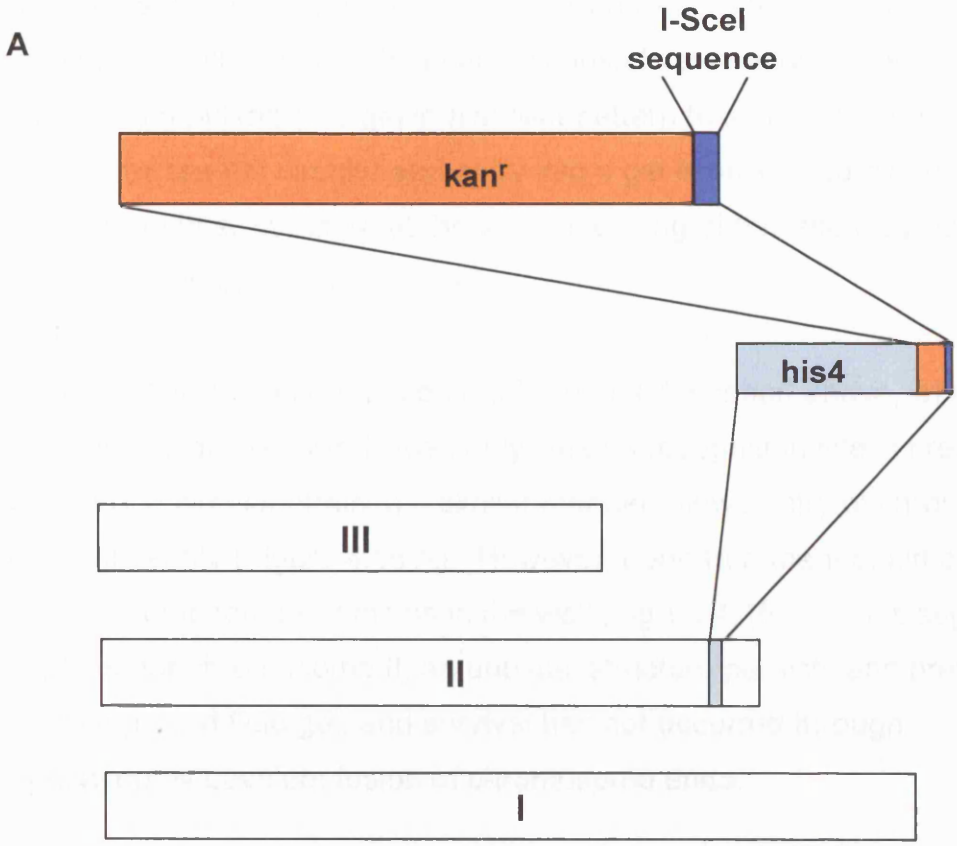
A potential reason for the reduced damage sensitivity and different patterns seen in X1 and X2 compared with a conventional circular survivor is that the chromosomes have remained linear with alternate structures, like persistent DNA branches or rolling circle replication intermediates at chromosome ends. The alternate structure at termini could explain why whole chromosomes or the terminal NotI fragments do not enter a pulsed field gel. If the sensitivity of the circular strains is due to the topology of the chromosomes, maintaining linear chromosomes, albeit with an alternate structure to wild type cells, might alleviate the damage sensitivity. To determine if the chromosomes in the new survivors are in fact circular, we carried out two experiments. Firstly we engineered a unique homing endonuclease site within the genome at a single locus to mechanically open a single chromosome. The second experiment we carried out was to γ-irradiate chromosomes within the agarose plugs used for pulsed field gel electrophoresis. This would allow us to look at all three chromosomes following induction of DNA double strand breaks. Introducing a break into a circular chromosome, be it through cutting with an enzyme or through γ-radiation, should allow entry into a whole chromosome pulsed field gel. However, if an alternate structure prevented entry into the gel, that structure may continue to prevent entry following cutting of the chromosome.

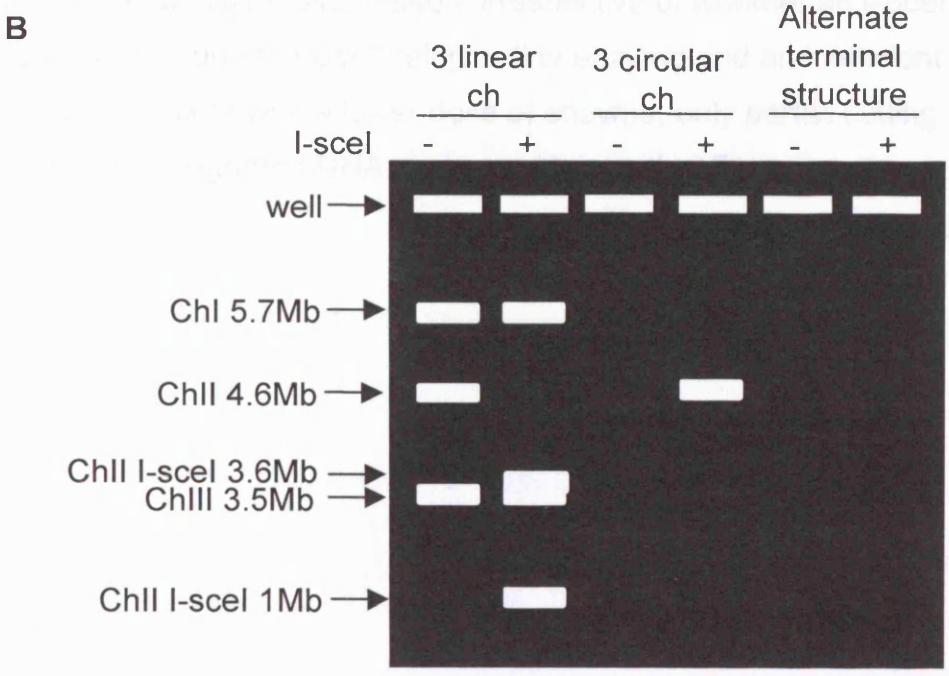
4.11.1.1 Opening chromosome II at an engineered I-SceI site

The recognition sequence for the homing endonuclease, I-SceI, was engineered into chromosome II of a linear strain, a circular strain and each survivor X1 and X2 (Figure 4.15 A). Presence of the site and cutting was verified by I-SceI digestion and restriction endonuclease digestion of genomic DNA followed by southern analysis (Appendix, A4). Digestion of genomic DNA from a linear strain with I-SceI should cut chromosome II into two fragments, one about 1Mb and the other about 3.6Mb in size (this larger fragment is indistinguishable from the 3.5Mb chromosome II band).

Figure 4.15 Schematic representation of method used to assess circularity of chromosomes by digestion with a uniquely engineered I-SceI site

(A) Engineering the I-SceI restriction site into the *his4* locus of chromosome II.
(B) Possible outcomes of I-SceI digestion followed by PFGE analysis of chromosomes with different structures. Digesting a linear chromosome would cut chromosome II into two discrete bands. Digesting a circular chromosome would linearise chromosome II, allowing entry into a pulsed field gel. Digesting a chromosome with an alternate structure at termini that prevents entry into a pulsed field gel may still not allow entry; the structure would still persist.


A
I-SceI sequence
kan^r
his4
III
II
I


B
3 linear ch
3 circular ch
Alternate terminal structure
I-sceI
- + - + - +
well
ChI 5.7Mb
ChII 4.6Mb
ChII I-sceI 3.6Mb
ChIII 3.5Mb
ChII I-sceI 1Mb

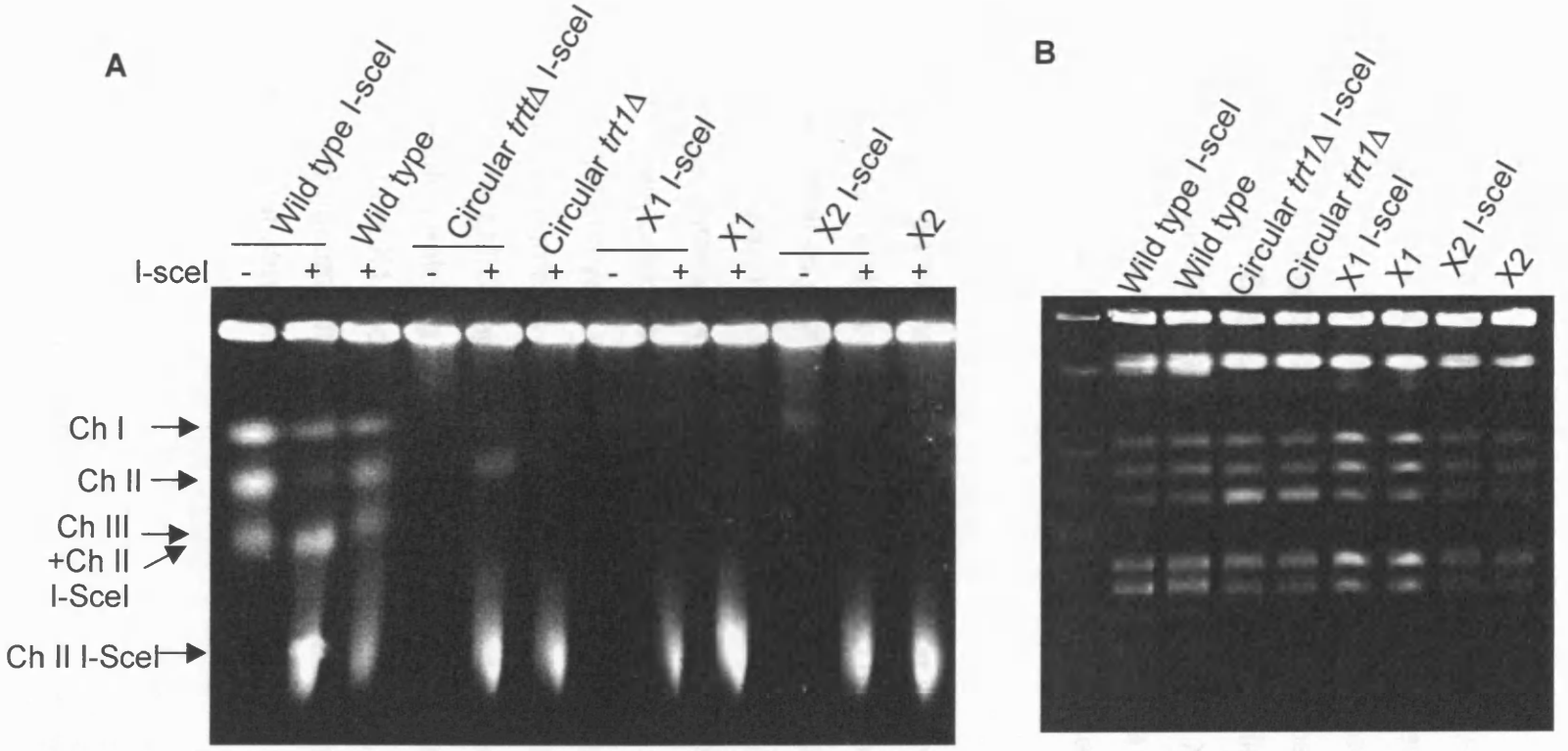
Disappearance of the 4.6Mb band of chromosome II would also be seen (Figure 4.15 B). I-SceI digestion should linearise a circular chromosome, allowing entry into the pulsed field gel. The chromosome would run as a single entity at 4.6Mb (Figure 4.15 B). If X1 and X2 do have circular chromosomes, cutting with I-SceI should cause linearisation of chromosome II and entry into a pulsed field gel in a similar pattern to a circular strain. If the chromosomes are not circular and entry into a gel is prevented by the presence of another structure at the termini, cutting at this site may not allow entry of the chromosome into the gel.

Figure 4.16 shows that chromosome II is cut, as described above, when a linear strain is digested with I-SceI, only when a recognition site is present. Treatment of a circular strain in a similar manner allows entry of chromosome II as a single entity (Figure 4.16 A). However, upon treatment of either strain X1 or X2, chromosome II remains in the well (Figure 4.16 A). This suggests that, at least for chromosome II, an unusual structure persists and prevents entry into a pulsed field gel, and survival has not occurred through circularisation by covalent fusion of chromosome ends.

In Figure 4.16, following treatment with I-SceI, an intense smear of low molecular weight DNA can be seen, irrespective of whether an I-SceI site is present. This is due to I-SceI being a dirty enzyme and an inefficient cutter. Following treatment with a lower dose of enzyme, only partial cutting is observed, and degraded DNA is absent (Appendix A5).

Figure 4.16 Pulsed field gel electrophoresis of chromosomes digested with I-SceI

(A) Chromosome II of a linear strain is cut into two fragments following I-SceI treatment, only when an I-SceI site is present. Treatment with I-Sce1 allows chromosome II of a circular strain with an engineered I-SceI site to enter a pulsed field gel, running as a single linear molecule. Treatment of X1 and X2 chromosomes containing engineered I-SceI sites does not allow entry into a pulsed field gel.
(B) NotI digestion of chromosomes shows equal loading of DNA in (A).


A
Wild type I-sceI
Wild type
Circular trt1Δ I-sceI
Circular trt1Δ
X1 I-sceI
X1
X2 I-sceI
X2
I-sceI - + + - + + - + + - + +
Ch I
Ch II
Ch III
+Ch II
I-SceI
Ch II I-SceI
B
Wild type I-sceI
Wild type
Circular trt1Δ I-sceI
Circular trt1Δ
X1 I-sceI
X1
X2 I-sceI
X2

4.11.2 Opening all three chromosomes by low dose γ-irradiation

Treating DNA in agarose plugs with increasing doses of γ-radiation prior to running on a pulsed field gel should introduce double strand breaks and should linearise circular chromosomes, allowing entry into a pulsed field gel. Chromosomes containing other structures may still be unable to enter a gel. As with digestion with I-SceI, treatment with γ-radiation allowed entry of chromosomes in a circular strain, but not in strains X1 or X2 (Figure 4.17). This again suggests that the survival mechanism in these damage resistant survivors is by a mechanism different to that of a sensitive telomerase deficient circular strain.

Intriguingly, following treatment of a 'normal' circular survivor with γ-radiation, only chromosomes I and II enter the gel (Figure 4.17). This has lead us to question if maintenance of chromosome III in a 'normal' *trt1*Δ survivor is in fact by a different mechanism to chromosomes I and II; a mechanism reminiscent to that of all three chromosomes in the damage resistant X1 and X2 survivors. Interestingly, when looking at SfiI digested chromosomes from a circular strain by pulsed field gel analysis, we would expect to see a band representing the fusion of terminal fragments of chromosome III upon probing with rDNA repeats as with the LMIC fragments of chromosomes I and II. However, we do not see the DNA entering the gel (Figure 4.18). This is reminiscent of all terminal fragments of X1 and X2 in a NotI pulsed field gel, further supporting the idea that chromosome III is maintained by a different mechanism in conventional circular strains.

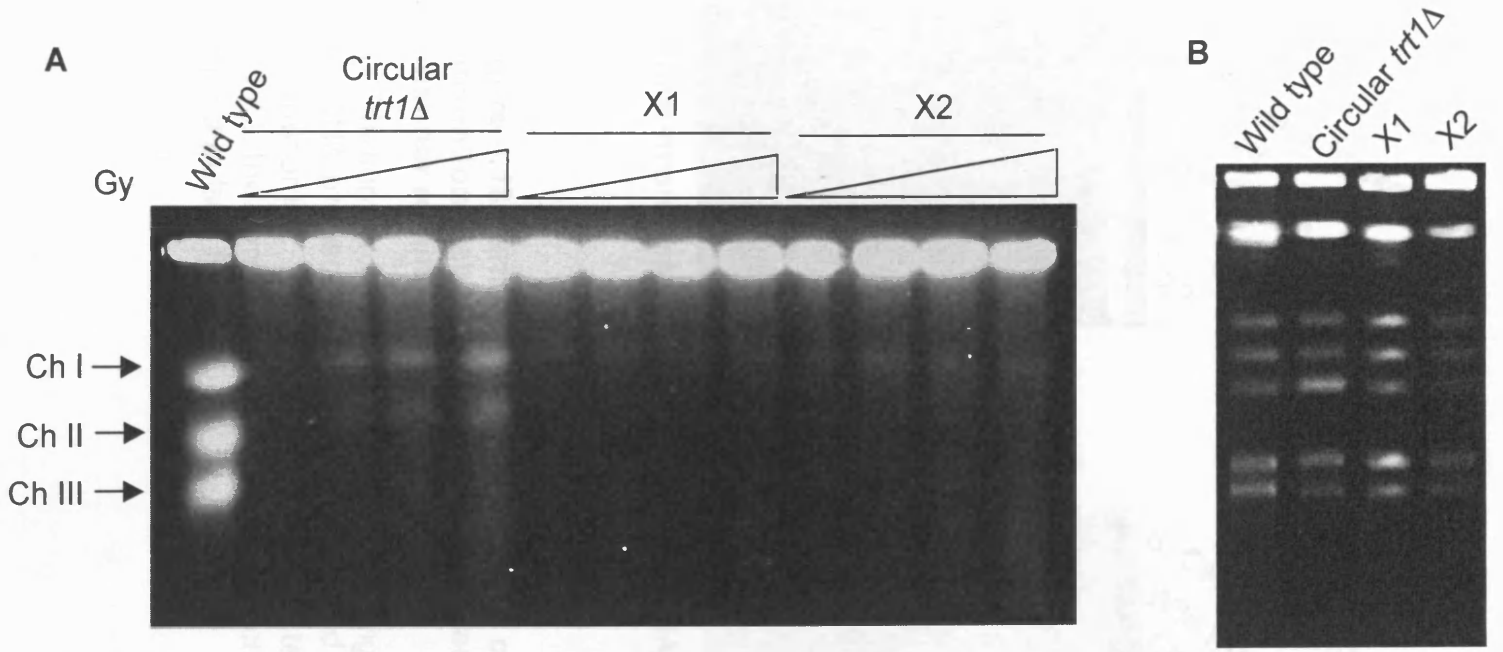


Figure 4.17 γ-irradiation of X1 and X2 chromosomes does not allows entry into a pulsed field gel

(A) Increasing amounts of γ-radiation allows entry of previously circular chromosomes as they are linearised by creating DSBs. Following treatment, chromosomes from X1 and X2 do not enter the gel.
(B) NotI digestion of chromosomes shows equal loading of DNA in (A).

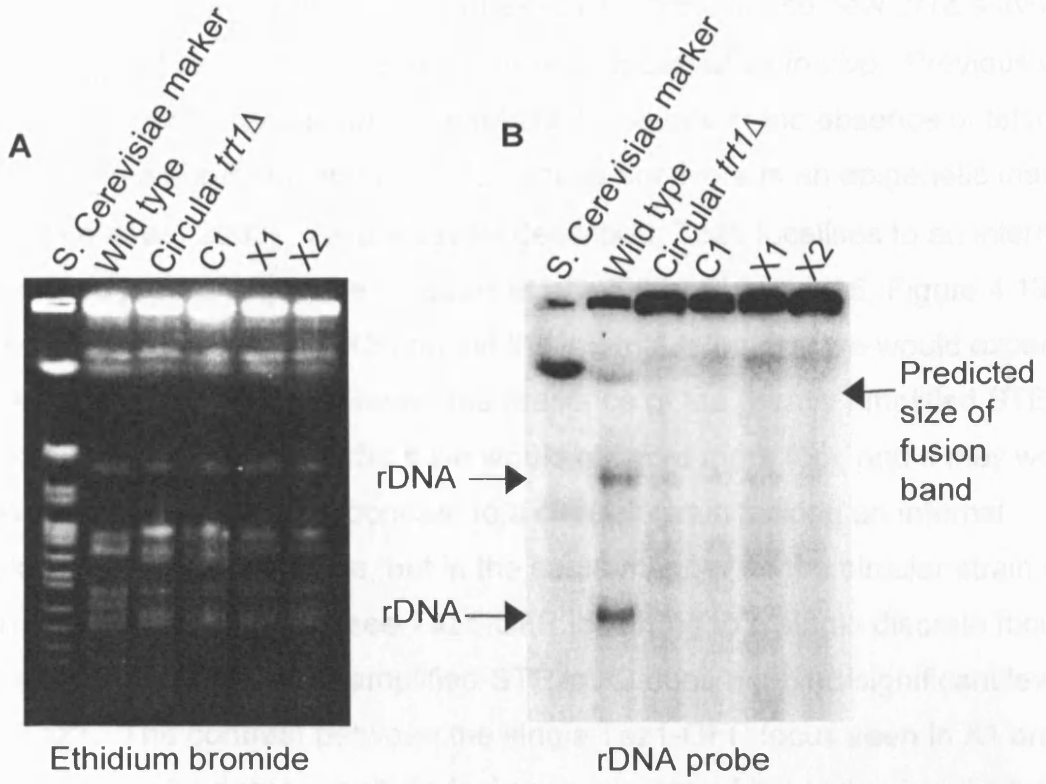


Figure 4.18 Terminal fusion fragments of chromosome III in a conventional circular strain do not enter a pulsed field gel in a manner reminiscent of X1 and X2

(A) Ethidium bromide stained gel of SfiI digested chromosomes. (B) rDNA probed Southern of SfiI digested circular *trt1*Δ chromosomes. In a similar manner to all terminal bands of X1 and X2, the terminal SfiI fusion bands of chromosome III do not enter a pulsed field gel.

4.12 Taz1-GFP localises to a single, discrete focus in X1 and X2

The role of Taz1 in end protection, telomere maintenance and survival following DNA damage led us to question its role in these new *trt1*Δ survivors. We tagged Taz1 using GFP to visualise its localisation *in vivo*. Previous work suggests Taz1 may also bind subtelomere regions in the absence of telomere repeats in a sub-population of *trt1*Δ circular survivors in an epigenetic manner (Sadaie et al., 2003). As previously described, Taz1 localises to an internal telomere repeat sequence ((Sadaie et al., 2003), Figure 3.16, Figure 4.19). Because strains X1 and X2 contain the internal telomere, we would expect to see focus formation. However, the presence of the greatly amplified STE sequences led us to wonder if we would observe more foci, and if they would also be more intense. In contrast to a circular strain lacking an internal telomere repeat sequence, but in the same manner as the circular strain with an internal telomere, we see Taz1-GFP localising to a single discrete focus (Figure 4.19). Thus, the amplified STE in X2 does not bind significant levels of Taz1. The contrast between the single Taz1-GFP focus seen in X1 and X2 and the much brighter, multiple foci seen in many of the cells of a wild type strain, further suggests any possible chromosome ends in X1 and X2 do not bind Taz1.

4.13 Disruption of *taz1*$^+$ in X1 and X2 does not change the STE pattern

To further investigate any possible role of Taz1 in the survival of X1 and X2 we looked at the effect of disrupting Taz1. Taz1 plays a role in suppressing recombination of telomeric and subtelomeric regions in fission yeast (Miller et al., 2006; Nakamura et al., 1998). Disruption of *taz1*$^+$ in X1 and X2 does not significantly alter the STE1 pattern (Figure 4.20). This is perhaps not surprising given that there is little, if any, telomere sequence in these strains. Linear *taz1*Δ strains have hugely elongated telomeres and single strand overhangs (Cooper et al., 1997; Tomita et al., 2003). It is likely the long single strand overhang initiates recombination events through invasion of other

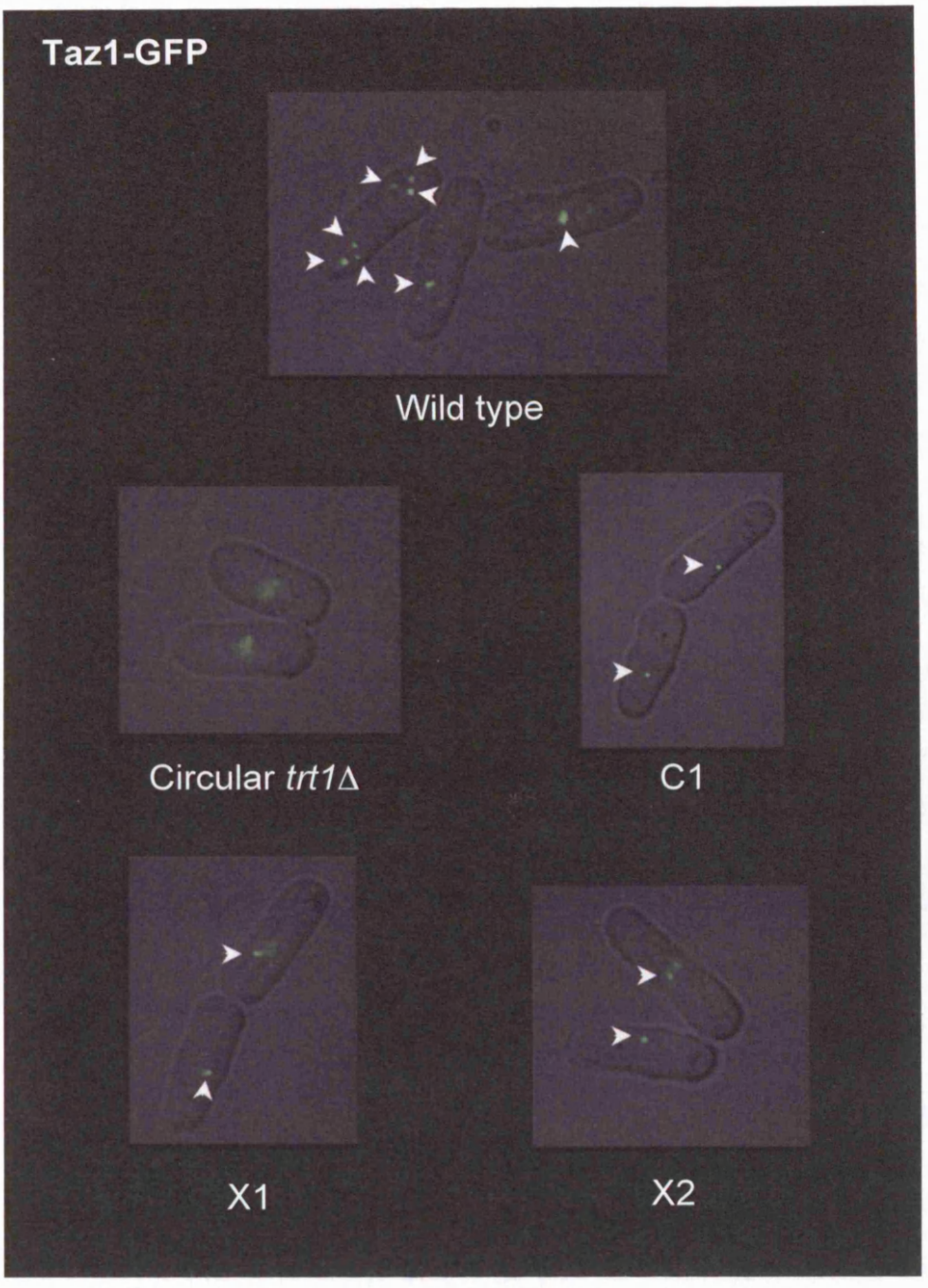


Figure 4.19 Taz1-GFP localisation in X1 and X2

(A) Wld type Taz1-GFP localisation. 1-3 foci can be seen with Taz1-GFP loaclising to clustered telomeres.
(B) Circular *trt1*Δ Taz1-GFP. Circular strains lacking telomeres do not show Taz1-GFP foci.
(C) Circular *trt1*Δ + internal telomere Taz1-GFP. A single focus is seen where Taz1-GFP localises to the internal telomere repeat sequence.
(D) X1 Taz1-GFP. A single Taz1-GFP focus can be seen.
(E) X2 Taz1-GFP. A single Taz1-GFP focus can be seen.

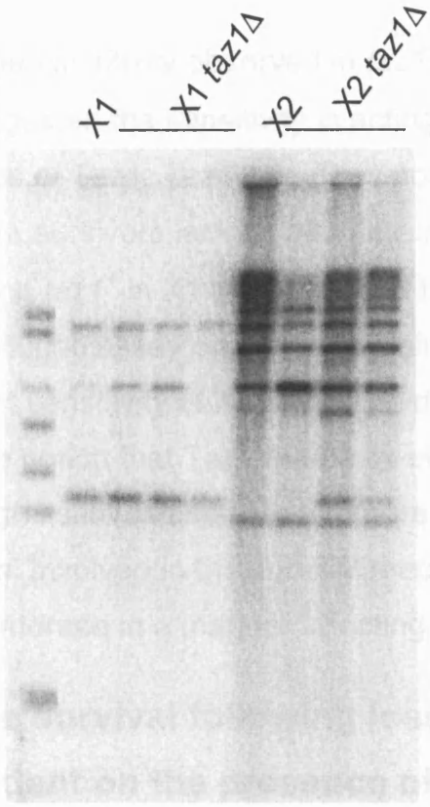


Figure 4.20 Disruption of *taz1*$^{+}$ does not cause STE rearrangements in X1 and X2

Southern analysis of NsiI digested genomic DNA. Hybridisation with STE1 probe demonstrates that Taz1 is not required to repress recombination in the sub telomeric sequences of X1 and X2.

telomeres. However, we demonstrated that X1 and X2 do not appear to have terminal telomere or subtelomere elements. Therefore, any overhang that may be present at a chromosome end would not involve these sequences and recombination involving these sequences would not be initiated.

4.14 Disruption of *taz1*$^+$ in X1 and X2 does not alter the damage sensitivity

The cause of the damage sensitivity observed in *taz1*Δ strains is not known. We have previously suggested the sensitivity is acting through dysfunctional telomeres caused by loss of Taz1; Taz1 loss does not affect the damage sensitivity of circular *trt1*Δ survivors lacking telomere repeats (Chapter 3.4). We wondered if disrupting *taz1*$^+$ in X1 and X2 would have any affect on damage sensitivity. A dilution assay on plates containing MMS demonstrates that Taz1 does not affect sensitivity of X1 and X2 to damage (Figure 4.21). This further supports the notion that Taz1 exerts its effect on the damage sensitivity of cells through its involvement in telomere maintenance. It also suggests that Taz1 is not involved in the survival mechanism used by X1 and X2 following loss of telomerase in a manner affecting the damage sensitivity.

4.15 X1- and X2-type survival following loss of telomerase is rare and not dependent on the presence of an internal telomere sequence

The appearance of survivors X1 and X2 came by chance while investigating the MMS sensitivity of circular telomerase negative survivors. The strains were created from a starting strain containing an internal telomere sequence, integrated into the *ura4* locus. This raises the question of whether the internal telomere is required for the appearance of this type of survival, and if not, if the internal telomere sequence increases the frequency of this survival mode. Out of 35 transformants, we followed 16 that were derived from the internal telomere strain and 19 derived from a strain without the internal telomere. All survived by the previously described mode of chromosome circularisation, irrespective of presence or absence of internal telomere. However, it is important to point out that in the transformation proceedure that uncovered the new mode of survival, one third of the transformants looked at survived by

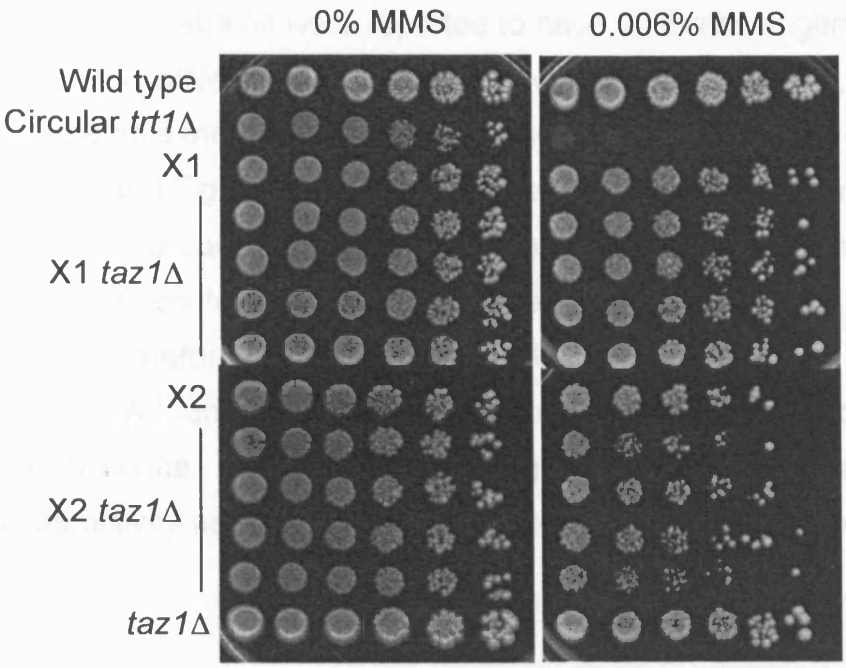


Figure 4.21 Disruption of *taz1*⁺ does not alter the damage sensitivity of X1 or X2

5-fold serial dilution assay on plates containing the indicated amount of MMS. Disruption of *taz1*⁺ from X1 and X2 does not affect the sensitivity to MMS.

the 'X' mode of survival. Whether this reflects some unrecognised difference in treatment of cells during survival is not known. Further analysis should be carried out to better define the frequency of survival by each of the 'X' modes of survival.

We did, however, obtain a 'circular' *trt1*Δ survivor from another lab (H1) that showed patterns reminiscent of X1, suggesting the 'X' mode of survival is not uncommon. We also received data from the same lab of a second 'circular' strain, H2. These strains were reported to have STE rearrangements and PFGE patterns unlike those of standard circular strains. Indeed, these patterns resemble those of survivors X1 and X2 (Compare Figure 4.22 A with Figure 4.4 B, and Figure 4.22 B with Figure 4.6). On further analysis of H1 we observed that this survivor also displayes only a mild damage sensitivity when grown on plates containing MMS in a similar manner to survivor X1 (Figure 4.22 C). We therefore deduce that survival of H1 is by a similar mechanism to that of X1 and X2, and therefore does not require the presence of the internal telomere sequence. It would be interesting to observe if H2 resembles X2 in damage sensitivity as it does with STE patterns and PFGE patterns.

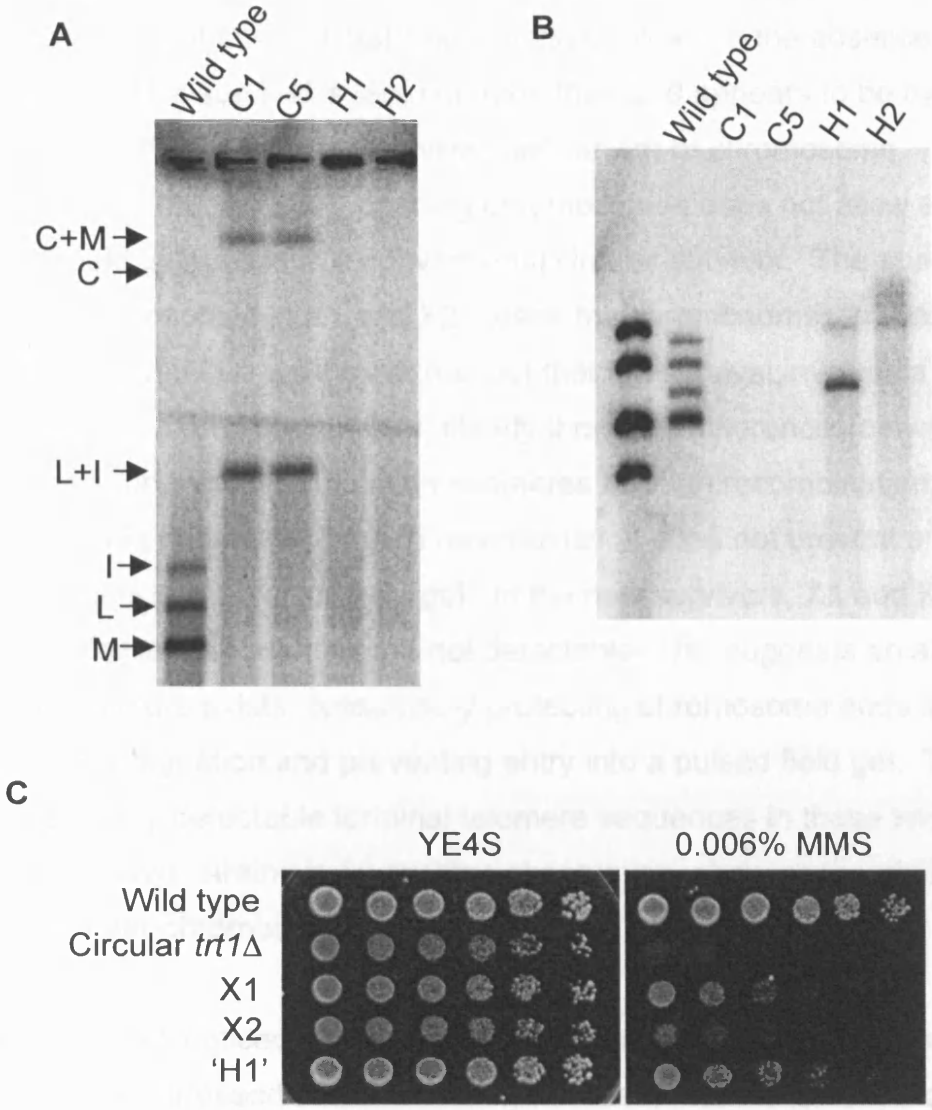


Figure 4.22 X1- and X2- type survival does not require the presence of the internal telomere

(A) 'Circular' *trt1*Δ survivors from the Cech lab. Some survivors, H1 and H2, display Not1 PFGE patterns different to a conventional circular survivor. These patterns resemble those of survivors X1 and X2.
(B) STE1 probed Southern of HindIII digested genomic DNA shows H1 displays STE1 patterns resembling X1, and H2 displays STE1 patterns resembling X2.
(C) H1 show a greatly suppressed damage sensitivity compared to a conventional circular survivor. This suppression resembles that of survivor X1.

4.16 Conclusion

In this chapter I have taken you through our understanding of a previously un-described mode of survival that exists in fission yeast in the absence of telomerase. This survival is less common than and appears to be by a mechanism different from the survival mechanism of chromosome circularisation; mechanically opening chromosomes does not allow entry into a pulsed field gel, unlike in a conventional circular survivor. The absence of circular chromosomes in X1 and X2 means the chromosomes in these strains must be linear. While we cannot rule out that the survival involves a recombination-based mechanism, clearly there are differences between these strains and survivors that maintain telomeres through recombination. Survival by maintaining telomeres through recombination does not prevent entry of chromosomes into a pulsed field gel. In the new survivors, X1 and X2, terminal telomere sequences are not detectable. This suggests an alternate terminal structure exists, presumably protecting chromosome ends from exonuclease digestion and preventing entry into a pulsed field gel. The absence of any detectable terminal telomere sequences in these telomerase negative survivor strains is an exciting observation, showing the ability to maintain linear chromosomes in the absence of telomeres.

X1 and X2 were noticed as being different from conventional circular strains due to their suppressed MMS sensitivity. Indeed, these strains are only mildly sensitive to the range of agents tested, suggesting the extreme sensitivity of circular strains is not due to lack of telomere repeats *per se*, but rather the topology brought about by the absence of telomeres. However, the further relief of MMS sensitivity brought about by reintroduction of telomerase, and proper telomeres, to X1 and X2 supports the possibility that telomeres are involved in the genomic DSB response.

X1 and X2 show defects in mitosis, and severe defects in meiosis in a similar manner to a circular strain. Thus, aspects of mitosis and meiosis may depend on having *bona fide* telomeres and not just chromosome linearity.

A particularly interesting observation was the amplification of different types of heterochromatic DNA in X1 and X2. X1 has survived with the amplification of rDNA repeats, X2 with subtelomeric elements. Whether this amplification is involved in the survival mechanism or the consequence of the new mode of survival needs to be addressed further. Results from preliminary experiments will be discussed in the next chapter.

The investigation of X1 and X2 also threw question onto the circularity of chromosome III in a conventional circular strain. In these strains, chromosome III fails to enter a pulsed field gel following γ-irradiation, similar to the failed entry of all the chromosomes of X1 and X2. Furthermore, the junction fragment in an SfiI digest fails to enter a pulsed field gel. Interestingly, it is chromosome III that contains the rDNA repeats. Thus, maintenance of chromosome III in a circular strain may reflect an analogous heterochromatin-based mechanism, to that occurring in X1 and X2.

The emergence of these novel strains could provide useful tools in the understanding of telomere function. They also demonstrate the ability of a cell to maintain linear chromosomes in the absence of terminal telomere repeats. The mechanism may reflect the ability of the cell to substitute telomeres with different DNA sequences and/or structures, albeit in a less efficient manner.

5 Discussion

Upon embarking on my thesis project, I set out to try and gain a better understanding of the role telomeres play in survival following DNA damage. To study this topic, I utilised telomerase negative strains that survive loss of all telomere and most of the telomere associated sequences by circularisation of each of the three chromosomes (Nakamura et al., 1998). In pursuing this study, two novel survivor strains emerged, also surviving in the absence of terminal telomere repeats. These strains were distinguished by their greatly suppressed damage sensitivity in comparison to conventional circular strains. The focus of a large part of my thesis turned to trying to gain a better understanding of the mode of survival of these strains and, in doing so, gaining a better understanding of the role telomeres play in surviving genotoxic insult.

5.1.1 Strains lacking functional telomeres display a range of defects

Upon careful analysis of strains harbouring circular chromosomes lacking telomeres, we observe that they display many defects, both during optimal vegetative growth and under extreme conditions such as in the presence of genotoxic insult or whilst undergoing meiosis. It is, at present, near impossible to distinguish whether it is the absence of terminal telomere repeats or the topology of the chromosomes that causes these defects, or indeed whether it is a combination of factors. Experiments adding telomeres to the cells must, by the nature of the system, be carried out with telomeres lacking ends on a plasmid or at an internal locus, or with the linearisation of one or more chromosomes through the addition of telomerase. While the internal telomeres form a telomere-like structure, it is also clear that they cannot represent true telomeres; they are not a 'true end'. Internal telomere repeats are known to be able to bind Taz1 and form a repressive chromatin structure ((Sadaie et al., 2003), Figure 3.16), however, it is also known that other proteins important in establishing a true telomere, such as Ku, are not recruited (Miyoshi et al., 2003). Another inherent part of the telomere

structure is the single strand overhang itself. Of course, an internal telomere sequence does not have this structure. Whether it plays an important role in the function of telomeres other than allowing telomerase to act at the termini and potentially promoting the formation of a terminal telomere structure, such as the T-loop, is not know. The emergence of the new type of survivor sheds at least some light onto the differentiation between the effects of chromosome topology and the absence of telomere repeats.

5.1.1.1 Strains lacking functional telomeres have chromosome segregation and septation defects

Initial observations suggest that even when grown under optimal conditions, cells harbouring circular chromosomes without telomeres have chromosome segregation defects. DAPI staining of chromosomes shows many aberrant patterns in circular strains, such as fragmented DNA and a 'cut' phenotype. Despite previous reports that circular *pot1*Δ survivors display a wild type morphology (Baumann and Cech, 2001), we observe elongated cells and DAPI staining suggesting chromosome segregation defects in a similar manner to circular *trt1*Δ strains. While *rad3*Δ*tel1*Δ circular strains are generally not elongated due to lack of damage and replication checkpoint function, many of the cells also display problems with chromosome segregation. This suggests that these problems are common to strains maintaining chromosomes as circles in the absence of telomeres. The topology of the chromosomes is likely to contribute, at least in part, to this defect. It is conceivable that circular chromosomes may form catenated or dicentric DNA molecules that, upon segregation to opposite poles, form chromosomes bridges and a 'cut' phenotype. However, the lack of telomere sequence itself cannot be ruled out as a contributory factor. In both fission yeast and budding yeast, the presence of a telomere sequences has been shown to aid with the segregation of circular plasmids (Longtine et al., 1992). This suggests, at least for plasmid DNA, telomeres are actually playing more of an active role in segregation rather than just maintaining the linearity. This may be through an association of telomere repeats with structures of the cell, such as the nuclear matrix, or with other chromosomes. Whether this phenomenon is carried through to genomic DNA also needs to be analysed further. A circular *trt1*Δ strain containing an internal telomere does not show

any improvement in cell viability, or reduction in chromosome segregation defects (data not shown). It may be possible, however, that the presence of an internal telomere sequence on each of the three chromosomes would be necessary to significantly aid in the segregation of circular chromosomes.

The presence of similar chromosome segregation defects in strains X1 and X2, which probably do not contain circular chromosomes but also lack terminal telomere repeats, makes a role of telomeres in chromosome segregation more attractive. However, it is also possible that the segregation defects are due to the presence of altered structures at chromosome termini. The inability of chromosomes to enter a whole chromosome pulsed field gel shows us that the chromosomes have formed an unusual structure, perhaps involving some kind of strand invasion or entanglement with other chromosomes. It is highly likely that these altered terminal structures may lead to defective segregation of chromosomes, causing the lagging and fragmented DNA as observed by DAPI staining. Cells lacking Taz1 display chromosome segregation defects when grown in the cold, a defect that is brought about by the entanglement of telomeres though to be caused by stalled replication forks that can not be resolved in the cold (Miller and Cooper, 2003; Miller et al., 2006). In a similar way, the structures formed at chromosome ends in X1 and X2 may also contribute to chromosome segregation problems.

Problems with chromosome segregation or the spindle assembly checkpoint were further demonstrated by the sensitivity of the circular strains to the microtubule depolymerising drug, TBZ. We did note, however, that sensitivity was less than that of the spindle checkpoint mutant, *bub1*Δ. Sensitivity to TBZ could indicate an inability to activate the spindle assembly checkpoint or a problem with microtubule dynamics. Bub1- and Mad2-GFP foci are observed in circular strains, suggesting activation of the spindle assembly checkpoint is not a problem. A proportion of the elongated cells observed in a culture may reflect activation of the spindle checkpoint as well as the Rad3-dependent checkpoint discussed in Chapter 1.4.1. In fact, despite a great reduction in elongated cells in circular *trt1*Δ strains lacking the Rad3 checkpoint function and circular *rad3*Δ*tel1*Δ strains, a small number of

elongated cells can still be found. Interestingly, budding yeast strains with defective telomeres through disruption of Ku70 and growth at 37°C activate a subset of spindle checkpoint pathways (Maringele and Lydall, 2002). Activation of spindle checkpoints through defective telomeres may be due to the formation of telomere fusions and the subsequent missegregation of dicentric chromosomes.

X1 and X2 are also mildly sensitive to TBZ, perhaps indicating a role of telomeres in the faithful segregation of chromosomes. Sensitivity was less pronounced than with the circular strains, perhaps reflecting a combined sensitivity in the circular strains from the lack of telomeres and the topological problem. Interestingly, unlike the other damaging agents studied, X2 was slightly less sensitive to TBZ than X1. It is conceivable that the hugely amplified subtelomeric sequences observed in this strain are able to partially compensate for the absence of telomere sequences.

The presence of multiseptated cells in the circular strains as well as X1 and X2 may suggest problems with the septation initiation network (SIN). Inappropriate activation of SIN can lead to cells with multiple septa. However, SIN mutants also often display mutinucleate cells, something that is not observed in these strains. The SIN is closely linked to the spindle assembly checkpoint (SAC) and so this might represent a general defect in anaphase.

5.1.1.2 Strains lacking functional telomeres undergo defective meiosis

Another defect previously observed in circular *trt1*Δ and *rad3*Δ*tel1*Δ strains is aberrant meiosis (Naito et al., 1998; Nakamura et al., 1998). I have shown that circular *pot1*Δ survivors also display a defect in meiosis. Telomere dynamics are very distinct during meiosis. Telomere clustering at the spindle pole body during early meiosis is observed in many eukaryotes. In fission yeast it has been shown to be important for correct meiotic chromosome segregation. Loss of Taz1 leads to dysfunctional telomeres, which is accompanied by a loss of telomere clustering during meiosis, disruption of the horsetail movement and subsequent meiotic chromosome segregation defects (Cooper et al., 1998). Meiosis involving circular chromosomes is

actually more severely altered than *taz1Δ* meiosis. Whether this reflects the complete lack of telomeres and therefore any remaining potential interaction involving telomeres during meiosis, or additional defects in circular strains caused by chromosome topology is not know.

A very attractive hypothesis that the lack of telomeres and therefore clustering at the SPB is contributing to defective meiosis in circular strains should be further addressed. I have demonstrated that the presence of a single, internally placed telomere tract that is able to bind Taz1 is unable to improve meiotic spore viability in a circular strain. Whether this telomere is able to cluster during meiosis and become involved in the telomere-led horsetail movement needs to be studied further. Also, the addition of telomere sequences onto each of the three chromosomes may be required to improve meiosis involving circular chromosomes. Previous work has demonstrated that different circular survivor populations arise with different meiotic clustering patterns (Sadaie et al., 2003). Type A derivatives show high frequencies of subtelomeric DNA associating with SPB in meiosis (>50% cells show association), type B derivatives show low association (<20%) and type AB show an intermediate association. The association correlates with the amount of STE retained prior to chromosome circularisation (Sadaie et al., 2003). However, no report was made about whether the ability to form a bouquet led to successful meiosis in these strains.

It is highly probable that meiotic recombination involving circular chromosomes would form dicentric chromosomes. Upon segregation of such dicentrics, breakage of DNA would occur, leading to the breakage-fusion-bridge cycle. This is a situation observed many years ago in maize by Barbara McClintock (McClintock, 1938; McClintock, 1939). In contrast to the situation in maize, in budding yeast it was found that meiotic divisions involving dicentric chromosomes did not cause chromosome breakage (Haber and Thorburn, 1984; Haber et al., 1984). It was postulated that the forces exerted by the centromere on a single microtubule were not strong enough to break apart a dicentric chromosome. In this way, the dicentric chromosome was transmitted to a single spore, and only upon sporulation and mitotic division did the dicentric chromosome break. The mitotic action of rotation of

the bud from the mother during cytokinesis was thought to provide an additional mechanical force strong enough to break a dicentric (Haber and Thorburn, 1984; Haber et al., 1984). Whether the situation in fission yeast resembles that of budding yeast or maize needs to be analysed further. Hence, while there is certainly a problem during meiosis prior to sporulation as observed by the aberrant asci formed, whether this is caused by the formation of dicentrics or alternative problems such as the lack of telomeres is not known.

As will be discussed in detail in the following section of this chapter, circular strains show a general sensitivity to DNA double strand breaks. This defect may also be reflected during meiosis. The reason for the damage sensitivity in circular strains is not fully understood, however, the presence of meiotic DSBs may also prove a problem for the cell. Meiosis involving circular chromosomes through disruption of *rad3*$^+$ and *tel1*$^+$ was worse than that involving circular chromosomes through disruption of *trt1*$^+$. This is likely to be due to the lack of checkpoint function in the circular *rad3*Δ*tel1*Δ strains. Indeed *rad3*Δ mutants show a reduction in spore viability (Murakami and Nurse, 1999; Shimada et al., 2002). It is likely that the decrease in spore viability of the *rad3*Δ*tel1*Δ meiosis compared with *trt1*Δ meiosis is due to the combined *rad3* defect and defects caused from undergoing meiosis with circular chromosomes. The presence of Rad3 during meiosis may be important not only for allowing repair of DSBs caused by meiotic recombination, but also for dealing with any breaks occurring from meiotic segregation of dicentric chromosomes, or indeed mitotic segregation upon re-entry to the mitotic cell cycle following germination of spores.

5.1.1.3 Functional telomeres are required for survival following DNA damage

A large focus of my thesis work has been trying to gain a better understanding of the role telomeres and chromosome topology play in survival following DNA damage, particularly in circular strains. Previous work from our lab implicated a role of telomeres in surviving DNA damage. Strains lacking the fission yeast telomere binding protein, Taz1, are sensitive to agents that induce DNA double strand breaks (Miller and Cooper, 2003). Evidence in other organisms

also suggests a role of telomeres or associated proteins in repair of DNA damage. Mice lacking telomerase display a general sensitivity to alkylating agents and γ-irradiation, but only in late generations when telomeres are shortened (Gonzalez-Suarez et al., 2003; Goytisolo et al., 2000; Wong et al., 2000). Telomerase negative human cell lines are sensitive to ionising radiation due to dysfunctional telomere structure, rather than length (Rubio et al., 2002).

The extreme damage sensitivity observed in circular fission yeast strains further supports a role for telomeres in surviving DNA damage. Furthermore, my observation that the sensitivity of circular strains is the same, in the presence or absence of Taz1 suggests the sensitivity of *taz1*Δ mutants stems from its role at telomeres. However, we cannot rule out the possibility that the very mild sensitivity of *taz1*Δ mutants to a high level of MMS would not be observed in the background of the extreme sensitivity of the circular strains to a low level of MMS. The existence of recombination-based linear *trt1*Δ *taz1*Δ survivors has allowed us to distinguish between the effects of telomere loss versus effects of the telomerase protein itself. Sensitivity of *taz1*Δ mutants is the same in the presence or absence of Trt1 when the chromosomes are maintained as linear molecules.

As with the previously described defects of circular strains, it is challenging to distinguish between the issue of chromosome topology and absence of telomere DNA in survival of DNA damage. There is a strong possibility that repair pathways cause problems to cells harbouring circular chromosomes. Resolution of recombination intermediates involving circular chromosomes could conceivably lead to dicentric and catenated chromosomes. However, these are structures that should be able to be resolved by the cell. Topoisomerases or Holliday junction resolvases should act to resolve these potentially lethal structures. However, we found that over-expression of either Top2, Top3 or Rqh1 did not prevent loss of viability following damage.

The presence of proteins involved in damage checkpoint and repair pathways at telomeres (Dahlen et al., 1998; Nakamura et al., 2002) led us to question a role of telomeres as a 'sink', providing a means for the cell to store and

stabilise the proteins until they are required in the relevant processes. I utilised a range of tools to address this possibility. I have shown that the presence of telomeres in high copy number as an episome do not aid in survival following damage. While internal telomere repeats are able to establish a telomere-like structure, they do not represent true telomeres as they lack ends as well as the single strand overhang.

We were able to demonstrate that damage checkpoint and repair processes are functional in circular strains lacking telomeres. The elongation phenotype observed in circular strains is indicative of checkpoint activation and is largely dependent on Rad3. Similarly, inactivation of the Rad3 checkpoint causes an increased loss of viability of the circular strains, both in an exponentially growing culture and in the presence of MMS. This demonstrates that telomeres are not required for the activation of a damage checkpoint. A repair assay of circular strains suggests that they are able to undergo repair following damage, however, due to the limitations of the assay, we were unable to confirm whether the repair efficiency is compromised in a circular strain. Inefficient repair would lead to an accumulation of damage, exhibited as an increased sensitivity.

Another possible role of telomeres in survival of DNA damage could be to arrange the chromosomes in a specific conformation or recruit them to a specific domain within the nucleus to allow efficient repair. In this case it may not be sufficient for a telomere to be present as an episome within the cell. In budding yeast, telomere clustering at the nuclear periphery is required for repair of subtelomeric regions (Therizols et al., 2006). While it was also shown that the clustering at the periphery was not required for the repair of more internal breaks within the genome, this does not exclude the possibility of a requirement for clustering in general DNA repair in fission yeast, or a similar, telomere dependent organisation of chromosomes not involving the nuclear periphery to be required for organising chromosomes for general repair of genomic DNA. Clustering to a domain within the nucleus may promote efficient repair by concentrating repair proteins to one area, or by promoting efficient repair of regions of similar sequences in close proximity by HR. It was previously shown that the efficiency of repair by NHEJ in

chromosomes lacking a homologous partner is reduced towards the telomere region, where HR and BIR increases. (Ricchetti et al., 2003). Repair of different regions of the chromosome may be favoured by different repair mechanisms.

We have shown that the sensitivity of circular *trt1*Δ survivors is partially suppressed by the addition of two terminal telomere sequences in the context of the ends of a chromosome. The re-addition of telomerase to a circular *trt1*Δ strain leads to the linearisation of chromosome III. This linear state is maintained through the addition of terminal telomere sequences to the chromosome. In a *taz1*Δ background, one might expect the deregulated telomere structure on the linearised chromosome III to contribute to the sensitivity as it does in a *taz1*Δ mutant with three linear chromosomes. However loss of Taz1 does not reverse the suppressed sensitivity achieved by reintroducing telomerase to circular strains. This suggests that the sensitivity of *taz1*Δ strains may be due to interactions between telomeres structures on different chromosomes. Alternatively, the low level of MMS used to observe the small amount of growth in the extremely sensitive circular strains might not be sufficient to confer additional sensitivity from the disruption of *taz1*$^{+}$.

Identification of the new *trt1*Δ survivors, X1 and X2, has given us a useful tool to help us understand the role of telomeres and chromosome topology in the survival following DNA damage. They were noticed as being different due to their suppressed MMS sensitivity. Indeed, these strains are only mildly sensitive to the range of agents tested. The analysis of X1 and X2 suggests that they have survived loss of terminal telomere DNA by a mechanism different to that of chromosome circularisation. They have maintained chromosomes without detectable terminal telomere repeats. X1 and X2 were isolated by deleting *trt1*$^{+}$ in strains harbouring an internal telomere tract. In addition to X1 and X2, this manipulation yielded 'normal' circular strains showing severe MMS sensitivity. Hence, the internal telomere tract is not sufficient to confer the 'X' phenotype. Furthermore, I have found that survivors isolated from the Cech lab that lack internal telomere repeats also show an 'X' phenotype, suggesting the internal tract is not only insufficient but

is also unnecessary. However, the mild sensitivity of X1 and X2 suggests that functional telomeres may play some role in surviving DNA damage.

The amplification of STEs in X2 suggested a possible role for these sequences in surviving DNA damage. Taz1 mediated heterochromatin formation extends to the subtelomeric regions (Kanoh et al., 2005; Sadaie et al., 2003). Thus, disruption of this subtelomere structure, as seen in *taz1*Δ mutants and circular strains, could cause damage hypersensitivity. However, while X1 has retained a small portion of STE, there is far less in comparison with X2. Both strains display a greatly suppressed damage sensitivity in comparison with standard circular survivors. However it is X1, the strain with only a small retention of STE, that shows the greatest suppression of damage sensitivity. Furthermore, disruption of *taz1*$^{+}$ in either strain fails to exacerbate the sensitivity. In these strains, analysis of the effect of Taz1 loss is not complicated by extreme damage sensitivity of the 'starting strain' as is is in standard circular survivors. The lack of STE rearrangements following loss of Taz1 and the presence of only a single Taz1-GFP focus in X1 and X2 (presumably localising to the internal telomere tract) supports the idea that Taz1 is not required to maintain structures that promote damage resistance in X1 and X2. The absence of any additional damage sensitivity following loss of Taz1 further emphasises that *taz1*Δ sensitivity occurs due to defective telomeres rather than loss of the protein itself. In these strains the result is not complicated by an extreme sensitivity to damage as observed with circular strains. It is likely that the role of Taz1 in survival following DNA damage is to maintain an amenable structure at chromosome ends.

Restoration of terminal telomere structures to all three chromosomes of X1 and X2 through reintroduction of telomerase completely alleviates damage sensitivity. This demonstrates that the mild sensitivity observed in these strains is due to the lack of telomeres and possibly the alternate structures formed at chromosome ends rather than the chromosome rearrangements that have occurred during survival.

Therefore, while telomeres appear to confer resistance to DNA damage, it is likely that this is by preventing structures that pose potential problems to cells while undergoing repair of damage.

5.1.2 Identification of two *trt1*Δ mutants with novel survival mechanisms

The identification of survivors X1 and X2 reveals a previously unrecognised mode of survival. These strains lack terminal telomere sequences and have amplified different types of repetitive, heterochromatic DNA- rDNA repeats or telomere associated sequences (STE). Initial observations suggested that survival was through chromosome circularisation. Chromosomes are unable to enter a PFG and telomere related sequences observed by Southern analysis are not terminal. However, further analysis has demonstrated that survival is by a mechanism different to the usual circularisation through intramolecular fusions seen in most *trt1*Δ survivors and other mutants lacking telomeres in fission yeast. The absence of intramolecular (or indeed intermolecular) fusions suggests that the chromosomes have remained linear, but have sustained some structural alteration that prevents their terminal fragments from entering PFGs.

In budding yeast lacking both telomerase and recombination pathways, rare survivors emerge that maintain chromosomes by the so-called PAL-mechanism (Maringele and Lydall, 2004b). These strains survive with greatly rearranged chromosomes often lacking detectable telomere signals. The linear chromosomes are maintained by the formation of large palindromes at chromosome ends. However, there are clear differences between the budding yeast PAL survivors and X1 and X2, suggesting the survival mode is not analogous and an alternate mechanism exists in fission yeast. While chromosomes from survivors X1 and X2 are unable to enter a PFG, chromosomes in PAL survivors are able to enter, suggesting different structures have formed at chromosome termini of the X-type survivors. Survival by the PAL mechanism requires disruption of the exonuclease, Exo1. The PAL mechanism involves both deletion of large parts of chromosome ends and duplication of other areas. We did not observe deletion of sequences past the STE.

Interestingly, two telomerase negative cell lines have been described in *Arabidopsis* with striking similarities to X1 and X2. Both cell lines lack terminal telomere repeats. Cell line A has undergone amplification of subtelomeric sequences that, according to the results of BAL-31 treatment, are not terminal. The other, cell line B, shows a high level of rDNA amplification (Watson et al., 2005). These rDNA rearrangements were initially observed in late generation telomerase deficient lines and attributed to continuous rounds of the 'breakage-fusion-bridge' cycle (Siroky et al., 2003). Survival of line B was suggested to occur with ongoing rounds of this cycle involving the rDNA repeats (Watson et al., 2005). The striking similarities between the *Arabidopsis* cell lines A and B and our fission yeast survivors X1 and X2 suggest survival in the absence of telomerase by a similar mechanism may occur from yeast to plant. However, we do not favour the hypothesis X-type that survival is by continued rounds of the breakage-fusion-bridge cycle.

The survival patterns exhibited by X2 bear striking similarities to Type I telomerase negative survivors in budding yeast. Both display amplification of subtelomeric DNA. However, in the type I survivor pathway, the subtelomere DNA is terminal, as can be observed by BAL-31 digestion (Zubko and Lydall, 2006) and is capped with short TG_{1-3} telomere sequences detectable by Southern analysis. I have not been able to demonstrate that any of the amplified subtelomere DNA in X2 is terminal, and no terminal telomere sequences have been detected. Another striking similarity is the retrotransposon style amplification of sequences in each organism. Upon disruption of telomerase in budding yeast, activation of the Ty1 transposable element is observed (Scholes et al., 2003). The Ty1 element has been shown to be involved in the mobilisation of the subtelomeric Y' elements in type I telomerase deficient survivors (Maxwell et al., 2004). The extensive spreading of the subtelomeric sequences throughout the genome of X2 is reminiscent of a similar retrotransposon-type amplification. However, it is not yet confirmed that either activation of a transposable element occurs in X2, or indeed that it is involved in amplification of the STEs.

The amplification of different types of heterochromatic DNA in X1 and X2 suggests that the heterochromatin nature of the DNA may play an important role in survival of these strains. It would be tempting to speculate that the repetitive nature of the sequences provides a means of maintaining and replenishing DNA at chromosome ends through recombination. However, there are significant differences between the behaviour of chromosomes from X1 and X2 compared with those from a telomerase negative survivor maintaining linear chromosomes through recombinational amplification of telomere DNA. Chromosomes with telomeres maintained by recombination enter a PFG (Appendix, A6). This could be because the telomeres are able to provide protection to the chromosome ends and are only involved in a recombination intermediate transiently in order to replenish the length of the sequence. The novel terminal sequences in X1 and X2 may not be sufficient to carry out the important function of a protective cap at chromosome ends unless involved in the recombination intermediate. In these cases, the structure may be more persistent, hiding ends from exonuclease digestion. Further analysis of these strains should be carried out to understand the role of heterochromatin in the survival mechanism. Initial results will be discussed in the following section. As discussed in greater detail in Chapter 1, *Drosophila* telomeres are formed from different types of sequence to conventional telomeres (Biessmann et al., 1992; Levis et al., 1993). The transposable elements that form the terminal sequences recruit the heterochromatin associated protein, HP1, to the chromosome ends (James and Elgin, 1986; Singh et al., 1991). Interestingly, the terminal sequence at chromosome ends in *Drosophila* is not required for the end capping function of the telomere. Flies with stable chromosomes lacking the transposable element sequences have been observed (Biessmann et al., 1990; Fanti et al., 1998; Levis, 1989; Mason et al., 1984). Despite the ability to maintain stable chromosomes lacking terminal repeats, binding of the telomere associated proteins to the terminal telomere structure is a prerequisite for chromosome stability in *Drosophila*, irrespective of terminal sequence (Cenci et al., 2003; Fanti et al., 1998; Perrini et al., 2004). This suggests that the heterochromatic nature of telomeres is sufficient to carry out the capping function of telomeres in *Drosophila*. Whether X1 and X2 represent a similar scenario in fission yeast will be interesting to observe.

5.1.3 The circularity of chromosome III in a conventional circular survivor

Our studies of the X strains have also led us to question the topology of chromosome III in a 'normal' circular survivor. The similarities we see between chromosome III in a circular *trt1*Δ strain and each of the three chromosomes in the two new survivors are striking. Analysing 'circularity' by whole chromosome pulsed field gel electrophoresis does not distinguish between circular chromosomes and other unusually structured DNA molecules such as recombination intermediates.

Analysis of NotI digested chromosomes by PFGE, the other common method of determining circularity of chromosome, allows the analysis of chromosomes I and II only. In these studies we have also carried out experiments that allow us to look at the circularity of chromosome III. In our experiments we have used PFGE of SfiI digested chromosomes which also allows analysis of chromosome III. Analysis by this method shows the rDNA-containing fusion fragment of a circular strain is unable to enter a pulsed field gel. This is a pattern we also see with the terminal 'LMIC' fragments of chromosomes I and II as well as the rDNA fragment of chromosome III in survivors X1 and X2. However, upon restoration of terminal telomere fragments, entry is permitted, suggesting the structure is alleviated. This is presumably because the formation of the unusual structure allows survival with loss of telomere protection on the specific chromosome, and the need for this is obviated upon addition of telomeres.

Mechanically opening the chromosomes by treatment with γ-irradiation should allow entry of all circular chromosomes into a whole chromosome pulsed field gel when the circle is broken. This was not observed in the chromosomes of X1 or X2, leading us to believe standard circularisation has not occurred and an unusual structure persists preventing entry into the gel. Surprisingly, we also observed this with chromosome III of a 'normal' circular strain.

Another interesting similarity observed between chromosome III of a circular survivor and all chromosomes in X1 and X2 was the linearisation upon

reintroduction of telomerase to each strain. We originally thought the reason for chromosome III linearisation was due to the presence of short telomere sequences interspersed within the rDNA repeats of chromosome III (Sugawara, 1989), combined with the continued reopening of these repeats whilst undergoing recombination. Indeed, the point of linearisation following addition of telomeres by telomerase is within the rDNA repeats as demonstrated by the presence of SfiI fragments hybridising with both a telomere and rDNA specific probe upon linearisation. However, the results described above suggest that chromosome III may not form a circular chromosome, but rather an alternate terminal structure like that in X1 and X2. The termini present in these chromosomes may provide an end for telomerase to dock and extend a telomere. It is particularly interesting to note that chromosome III contains the rDNA repeats at a subtelomeric location and survivor X1 has amplified rDNA repeats onto each chromosome at a 'terminal' region. It is probable that maintenance of these chromosomes is by a mechanism involving this repetitive, heterochromatic DNA. Presumably, the STE amplification observed in survivor X2 may be involved in chromosome maintenance a similar manner.

Some observations from a previous study could be explained by invoking X-type survival. In *trt1*Δ survivors cultured in liquid over a prolonged period (22 days), rearrangement of STEs was observed, as was the disappearance of the 'L+I' and much of the 'C+L/M/I' fusion bands (Baumann and Cech, 2000). It may be that these terminal phenotypes represent the emergence of this alternate type of survivor. While growth of survivors X1 and X2 is slow compared with wild type strains, there is a slight growth advantage compared with a circular survivor. It is conceivable that, while these survivors may be relatively rare compared with circular survivors, and therefore only observed occasionally when selected on plates, selection in liquid media favours the slightly faster growing mutants, and emergence of these survivors is observed after a period in liquid. Further analysis should be carried out to determine the frequency of appearance of these survivors and indeed if the survivors observed with STE rearrangement and loss of the 'LMIC' bands from culturing telomerase negative strains in liquid represent survival by a similar mechanism to X1 and X2.

The nature of the maintenance of chromosomes in the absence of terminal telomeres in X1 and X2 is at present only speculative. As described earlier, a recombinational mode of survival is an attractive model, but this recombination clearly differs from that seen in fission yeast lacking Taz1 and Trt1, and probably differs from budding yeast type I and II survivors. In organisms from yeast to plants and mammals (Bucholc and Buchowicz, 1995; Horowitz and Haber, 1985; Louis and Haber, 1990; Ogino et al., 1998; Regev et al., 1998; Underwood et al., 2004; Yeager et al., 1999) a 'rolling circle' mode of telomere amplification has been described. In these situations, extrachromosomal telomere DNA is thought to act as a template for amplification of telomere sequences through a recombination mediated pathway. It may be possible that a similar amplification of other repetitive sequence such as the rDNA or subtelomeric DNA occurs. It is conceivable that the continual presence of such structures at chromosome ends could prevent the entry of chromosomes and terminal fragments into a PFG. Perhaps a similar structure may be present at chromosome ends in strains X1 and X2, preventing entry into a PFG, and that this explains the lack of entry of DNA from 'X' strains. A structure such as this may also protect chromosome termini from digestion by BAL-31 nuclease. However, in budding yeast this is not the case; in strains sustaining rolling circle-mediated telomere maintenance, chromosomes and terminal NotI fragments are able to enter a PFG.

Another possibility that we have not investigated is that strains X1 and X2 do harbour circular chromosomes, but sustain additional structures at the fusion junctions, that prevent entry of these fragments into a PFG. The presence of repetitive DNA sequences, possibly as inverted, yet imperfect, repeats upon chromosome circularisation, may promote the formation of a cruciform-like structure. Resolvases may, theoretically be able to eliminate these structures. Elimination of these structured from the chromosomes may account for some of the change in size observed in chromosomes following linearisation through reintroduction of telomerase. Complete elimination may cause lethality due to the essential nature of some of the sequences. I think this is an unlikely scenario, but one that should perhaps be addressed. The

substantial decrease in damage sensitivity of a conventional circular strain following the addition of telomere repeats to chromosome III may support this model

From my analyses, I propose that survival of X1 and X2 in the absence of telomerase has occurred by a novel mechanism, utilising the amplification of heterochromatic sequences, either rDNA or STEs, to maintain linear chromosomes in the absence of telomeres. In a similar manner, chromosome III, containing the rDNA repeats at a subtelomeric location, is maintained using this mechanism in an otherwise circular strain. While survival by this mechanism proves problematic to a cell, the severity is decreased in comparison to survival by chromosome circularisation. However, the challenge of adequately amplifying these sequences to different chromosomes may mean survival by chromosome circularisation is more common.

5.2 Unresolved data and future perspectives

The work in my thesis has opened many doors for further research. I have touched on a range of defects displayed in strains lacking functional telomeres, either through chromosome circularisation or surviving by a novel mechanism in the absence of telomeres.

We still have a long way to go in understanding the cause of the defects observed in the survivors. In studying the role of telomeres through the use of circular survivors we have an unavoidable problem; the added issue of chromosome topology. Genetic manipulation of circular strains is difficult and augmented by the defective meiosis; rescue of diploids containing only circular chromosomes is near impossible, azygotic sporulation of such diploids does not occur (data not shown), and therefore tetrad analysis is not a possibility.

As for circular survivors, genetic analysis of X1 and X2 is difficult. Defective meiosis hampers genetic analysis. The relatively rare emergence of survivors utilizing X1- and X2- type mechanisms further complicates the problem. At present we are not aware of a situation whereby we can select for survival by

this mechanism. We are attempting to induce survival under MMS selectio,, expecting that the greatly reduced lethality conferred by X survival will tip the survival mode in this direction. We have also attempted to reverse X survival- to disrupt the underlying mechanism and force X cells to undergo chromosome circularisation. It would be interesting to see what are the requirements of survival by these methods. We have attempted to delete the *pot1*$^{+}$ gene in X strains, as Pot1 is thought to be essential for linear chromosome maintenance. Interestingly, a recent report shows that in budding yeasr type I and type II survivors, the usually essential Cdc13 is dispensable for growth (Larrivee and Wellinger, 2006).

5.2.1 Sensitivity of circular strains to DNA damaging agents

Another route we embarked upon to gain a better understanding of the damage sensitivity of circular strains was to carry out screen for high-copy number suppressors. However this proved problematic. We were reluctant to carry out the screen in the presence of MMS due to the mutagenic nature of the drug, and the sensitivity of the strains when treated in an acute fashion proved too mild to allow robust selection. While a high-copy screen was not a feasible option, a mutagenic screen might be a possible. When circular *trt1*Δ strains were grown on media containing MMS for an extended period (7 days), it was notable that suppressors emerged at a far higher frequency than any from the *rad3*Δ mutant. It would be interesting to analyse these suppressor strains in greater detail and to carry out a larger scale mutagenic screen on circular survivors, and may cast further light onto the basis of the damage sensitivity of circular strains.

5.2.2 Analysing the heterochromatic requirements in the new telomerase negative survivors

Clearly, an important future step to continue the work from this thesis is to understand the mechanism of survival of the new telomerase negative survivor strains, X1 and X2. The amplification of different types of heterochromatin poses an interesting possibility for the method of survival of these strains. To investigate the role of heterochromatinisation, we looked at the requirement for the histone methyltransferase, Clr4, in these strains.

Initial results have been contradictory. While some of the transformants observed display the same pulsed field patterns and damage sensitivity as X strains containing Clr4, some transformants showed increased damage sensitivity. Clones emerged with varying levels of increased damage sensitivity (Figure 5.1 A and data not shown). Those with increased sensitivity (actually to a level equivalent to that of a circular strain) display pulsed field gel patterns reminiscent of a strain with circular chromosomes (Figure 5.1 B). Hence, the disruption of heterochromatin structure may prevent survival by these novel methods. In these cases, survival switches to the mode of chromosome circularisation as chromosome ends are de-protected and undergo fusion. The clonal variation observed may be due to Clr4 being involved in heterochromatin establishment rather than maintenance (Ivanova et al., 1998; Nakayama et al., 2001). Further analysis into the involvement of heterochromatin in these new modes of survival may help us gain deeper insight into the survival mechanisms employed by these strains in the absence of telomerase. Further genetic analysis and/or the use of drugs such as trichostatin A to disrupt heterochromatin may be useful tools in these analyses. An understanding of the involvement of heterochromatin in these new modes of survival may help us gain deeper insight into the survival mechanisms employed in the absence of telomerase.

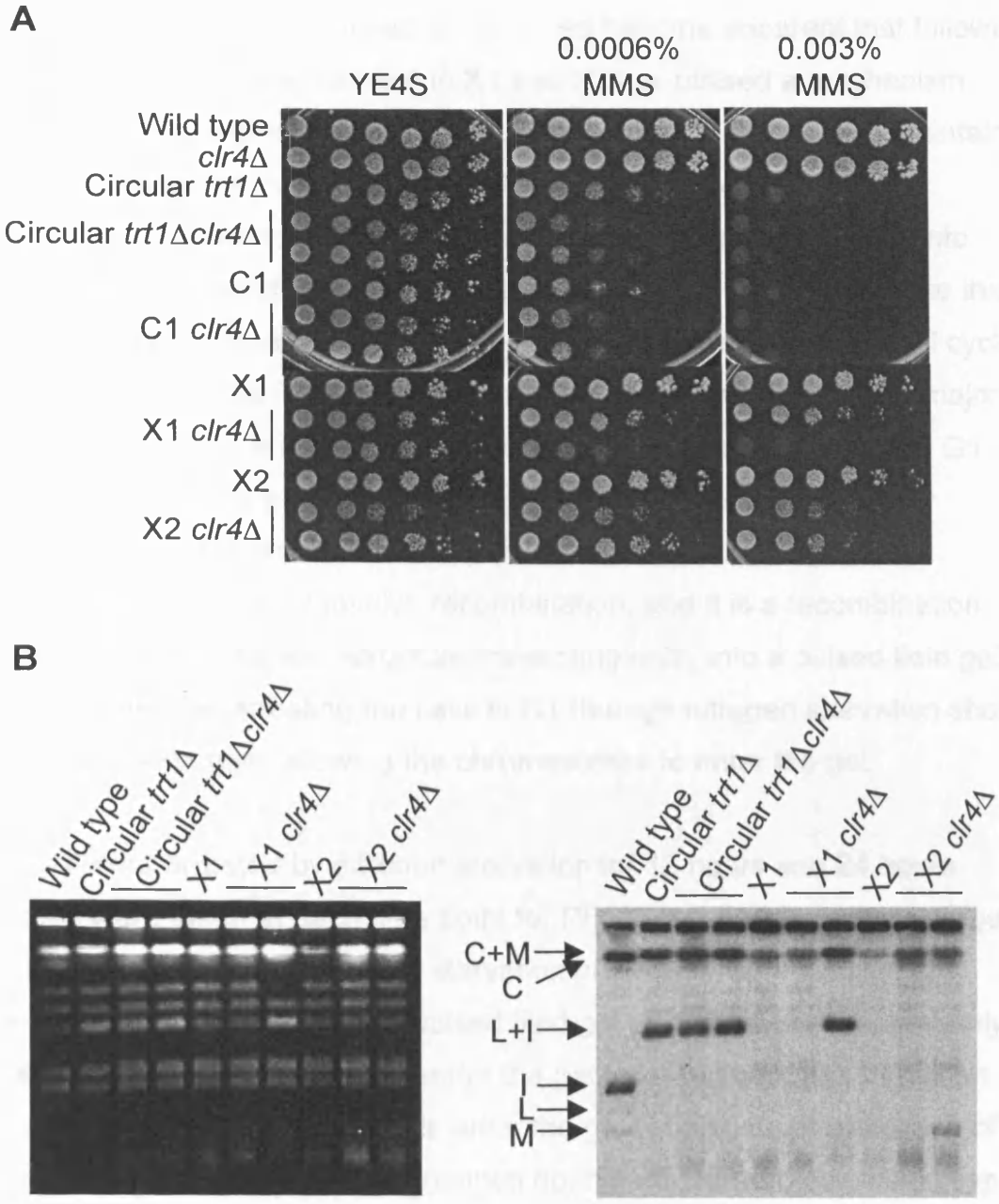


Figure 5.1 Role of Clr4 in chromosome maintenance in X1 and X2

(A) 5 fold serial dilution assay of *clr4*Δ mutants of different *trt1*Δ survivors. Some X1 and X2 clones lacking Clr4 show an increased damage sensitivity.

(B) '(LM)IC' probed Southern of Not1 PFGE demonstrates the emergence of fusion bands in the X1 and X2 *clr4*Δ mutants with increased damage sensitivity.

5.2.3 Nitrogen starvation allows entry of some DNA into a pulsed field gel

From the experiments discussed so far, it has become apparent that following disruption of telomerase, survival in X1 and X2 has utilised a mechanism different to those previously described. One possibility is that cells maintain linear chromosomes through a recombination based method in which recombination intermediates are constantly present, prohibiting entry into gels; the abundance of repetitive sequences in X1 and X2 may facilitate this. In fission yeast, modes of DNA repair are regulated throughout the cell cycle. In the G2 stage of the cycle, which predominates in fission yeast, the major mode of DSB repair is homologous recombination. However, during a G1 arrest, a stage that is normally brief in fission yeast, the mode of repair switches to NHEJ (Ferreira and Cooper, 2004). Should the survival mechanism of X1 and X2 involve recombination, and it is a recombination intermediate that creates a structure preventing entry into a pulsed field gel, it is conceivable that arresting the cells in G1 through nitrogen starvation should alleviate the structure, allowing the chromosomes to enter the gel.

Cultures were arrested by nitrogen starvation for 12 hours and 24 hours. Samples were taken at each time point for PFGE and FACS analysis. Figure 5.2 shows that following nitrogen starvation of X1 and X2, a single, high molecular weight band enters a pulsed field gel. It seems unusual that only this single, discrete band should enter the gel following nitrogen starvation. If the reason the DNA is now able to enter the gel is because of alleviation of a structure that is unable to be maintained during a G1 arrest, one would expect all three chromosome to enter the gel. Another possible reason for entry in this manner is that the process of arresting cells in G1 causes cells to die, leading to chromosome breakage and degradation of DNA. With chromosome I being the largest, we might expect this chromosome to incur breaks more frequently. However, I view this as unlikely. We have already shown that creating double strand breaks through γ-irradiation does not allow entry of chromosomes, and so it is unlikely breaks caused through nitrogen starvation stress would allow entry of chromosomes into a gel.

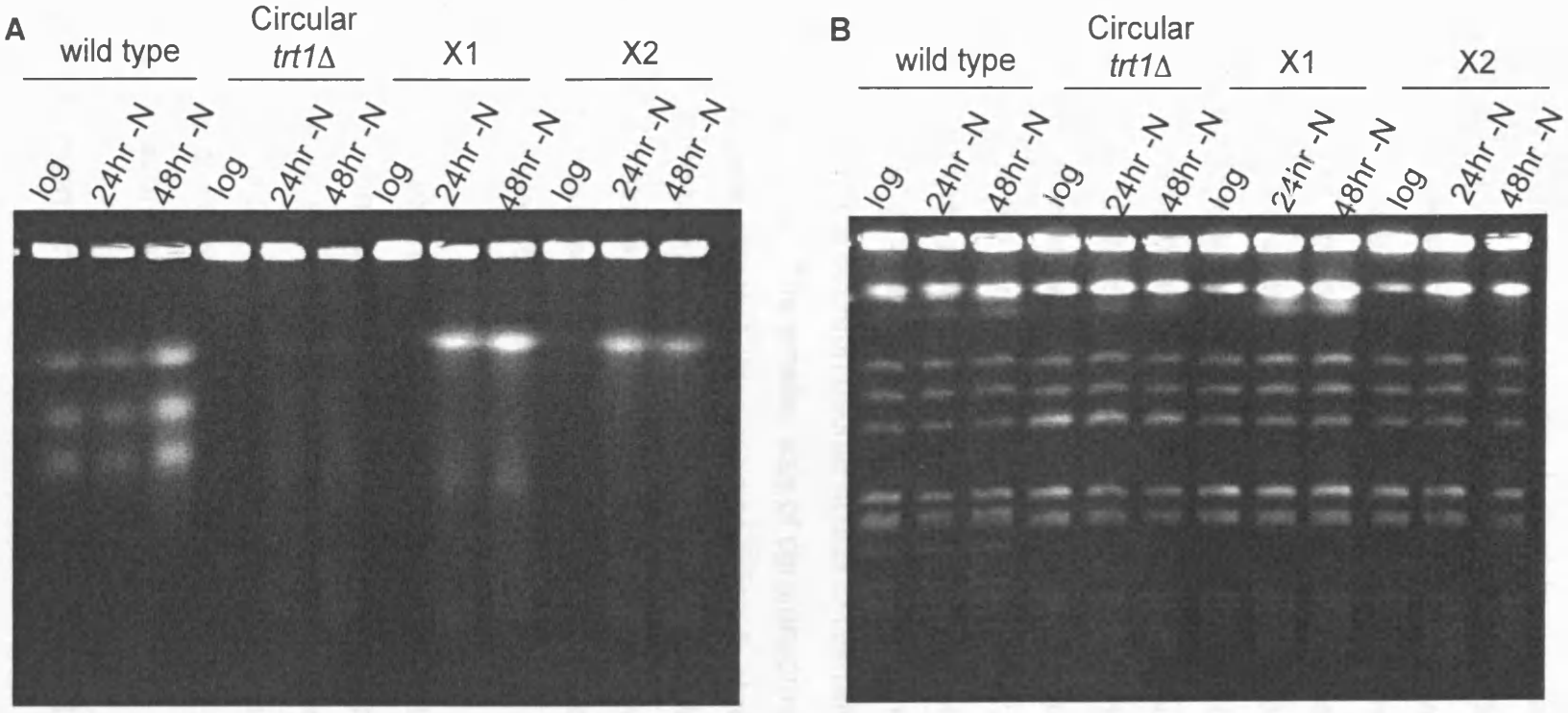


Figure 5.2 G1 arrest allows entry of a single band into a pulsed field gel in X1 and X2

(A) G1 arrest through nitrogen starvation. A single band is seen to enter a pulsed field gel in strains X1 and X2.
(B) NotI digestion of chromosomes demonstrates equal loading of DNA in (A).

Following 'linearisation' of chromosomes upon reintroduction of telomerase, we observed the chromosomes to have undergone extensive chromosomal rearrangements. Indeed, chromosome II in both strains was always increased in size and ran at a molecular weight approaching the size of chromosome I. It is conceivable that the single band observed upon G1 arrest could represent both chromosomes I and II. However if this were the case, is seems unusual that the band is not more diffuse, especially given the variation in size of chromosomes upon reintroduction of telomerase. We also have to question the absence of a band representing chromosome III. In strain X1, we see a diffuse smear at the size range of chromosome III (Figure 5.2). Following linearisation of chromosome III in circular strains, we often observe the size of chromosome III to be diffuse within an individual clone, and variable in size between different clones. As discussed in Chapter 3, this is possibly due to the variation in rDNA repeat number, the new conformation of the chromosomes promoting recombination between the inverted repeats. It may be that the diffuse smear in X1 following G1 arrest also represents a huge variation in size of chromosome III due to rearrangements occurring in this altered topology. The smaller size of chromosome III may allow better resolution of these different sizes in comparison to chromosomes II and III. Alternatively, the smear could represent broken DNA following nitrogen starvation. In a similar manner, strain X2 may also have a diffuse smear representing chromosome III, but in this case it is too diffuse to observe by pulsed field gel electrophoresis.

It is also conceivable that if recombination is involved in maintenance and protection of chromosome ends in X1 and X2, disrupting this structure via G1 arrest may cause the strains to switch to a circular chromosome form of survival. If this is the case we would not expect to see entry of chromosomes enter a whole chromosome PFG. Further analysis by NotI PFGE should be carried out to address this possibility.

5.2.4 Topoisomerase II overexpression and X1/X2 survival

Another interesting recent observation that may also give us a deeper understanding into the new telomerase negative survival mechanisms is the result of over-expression of topoisomerase II. Transformation with a plasmid

containing thiamine repressible *top2* leads to significantly decreased viability of X1 and X2, even in the repressed state (where a small amount of expression may occur) (Figure 5.3). Top2 is involved in the alleviation of DNA structures such as catenanes. It is possible that structures at the chromosome termini may act as substrates for Top2. Upon over-expression of Top2, these structures may be resolved, leading to de-protection of chromosome ends, fusion, and cell death. It would be interesting to determine whether the transformants that emerge still maintain the same topology, or if they have converted to the circular chromosome form of survival.

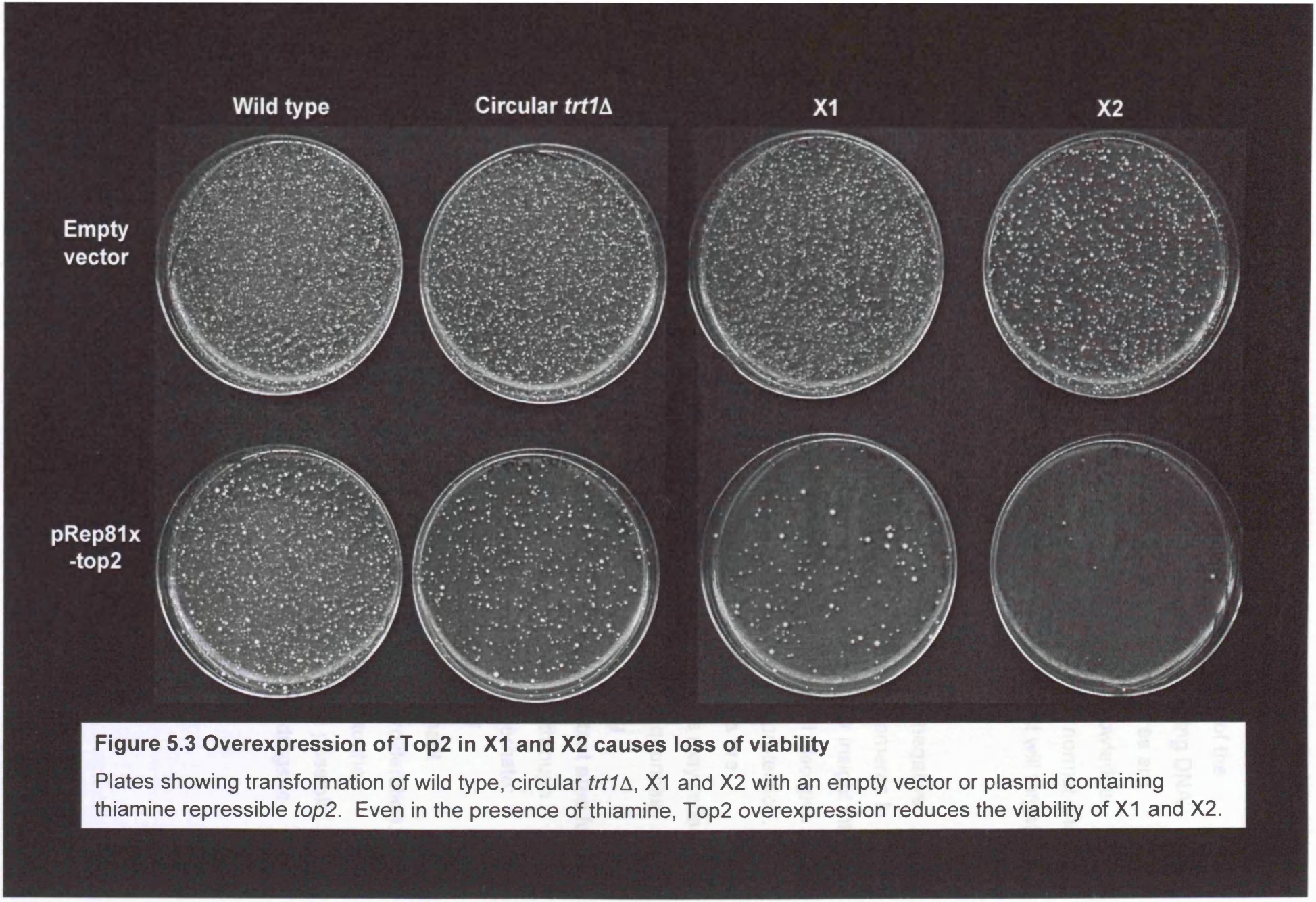


Figure 5.3 Overexpression of Top2 in X1 and X2 causes loss of viability

Plates showing transformation of wild type, circular *trt1*Δ, X1 and X2 with an empty vector or plasmid containing thiamine repressible *top2*. Even in the presence of thiamine, Top2 overexpression reduces the viability of X1 and X2.

5.3 My work in the bigger picture

Telomeres play an important function in maintaining the integrity of the genome. By further uncovering of the roles of telomeres in surviving DNA damage, we will gain a better understanding into the how telomeres are involved in maintaining the integrity of the genome. A deeper knowledge should give us insights into the differences between telomeres in normal and cancer cells, providing a better basis for developing therapies that will target cancers more specifically and effectively.

Understanding the survival mechanism of these new telomerase negative survivors should give us a better understanding of the roles of telomeres in different cellular processes. Already they have given us a greater insight into the role of telomeres in survival following DNA damage. They will also give us a better understanding of the requirements for chromosome end protection. Here we have a situation whereby seemingly linear chromosomes are maintained in the absence of telomere repeats. The mechanisms may reflect the ability of the cell to substitute telomeres with different DNA sequences and/or structures, albeit in a less efficient manner. Understanding mechanisms of survival in the absence of telomerase is an important area for cancer biology. While about 90% of cancers maintain telomeres through reactivation of telomerase, the remaining 10% survive through alternate mechanisms. While telomerase reactivation is the major mode of chromosome end maintenance in cancers, it occurs late in malignant transformation of tumours. Therefore understanding continued proliferation in the absence of telomerase may give us a better understanding into the processes occurring during tumorogenesis. Understanding each possible mechanism thoroughly should lead to a more informed design of drugs to treat cancer.

Appendix

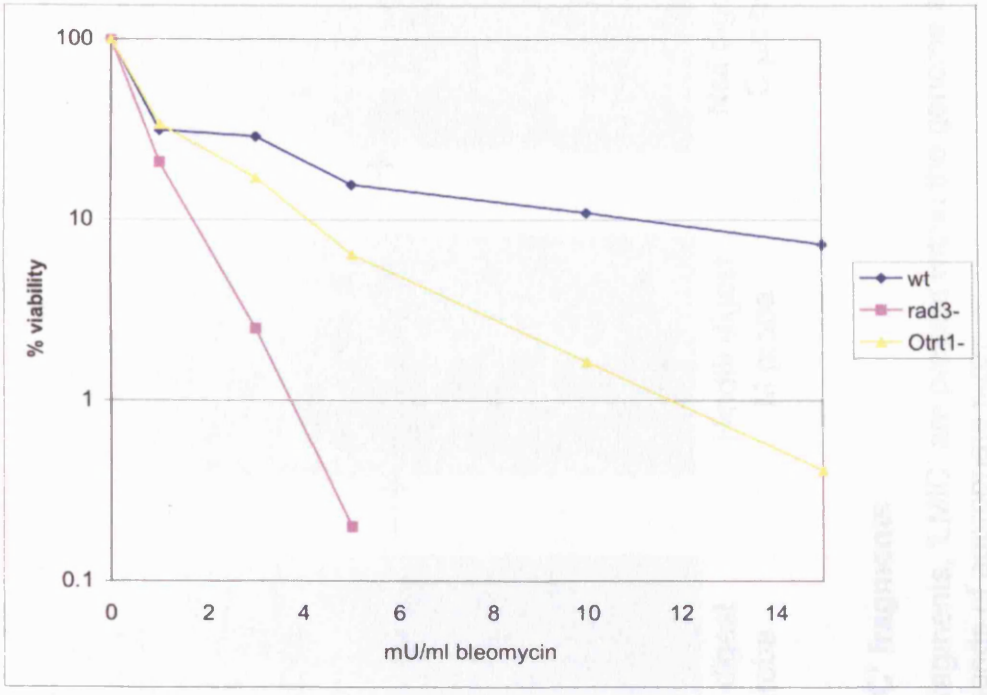


Figure A1 Circular strains are mildly sensitive following acute treatment with bleomycin

Following a 3 hour treatment with bleomycin at the indicated dose, 500 cells were plates onto rich media and colonies allowed to form. Viability was plotted as the number of colonies formed following treatment, compared with the number of colonies formed with no treatment. Treatment in this way causes loss of viability in a circular *trt1*Δ strain, but to a lesser degree than the *rad3*Δ checkpoint mutant.

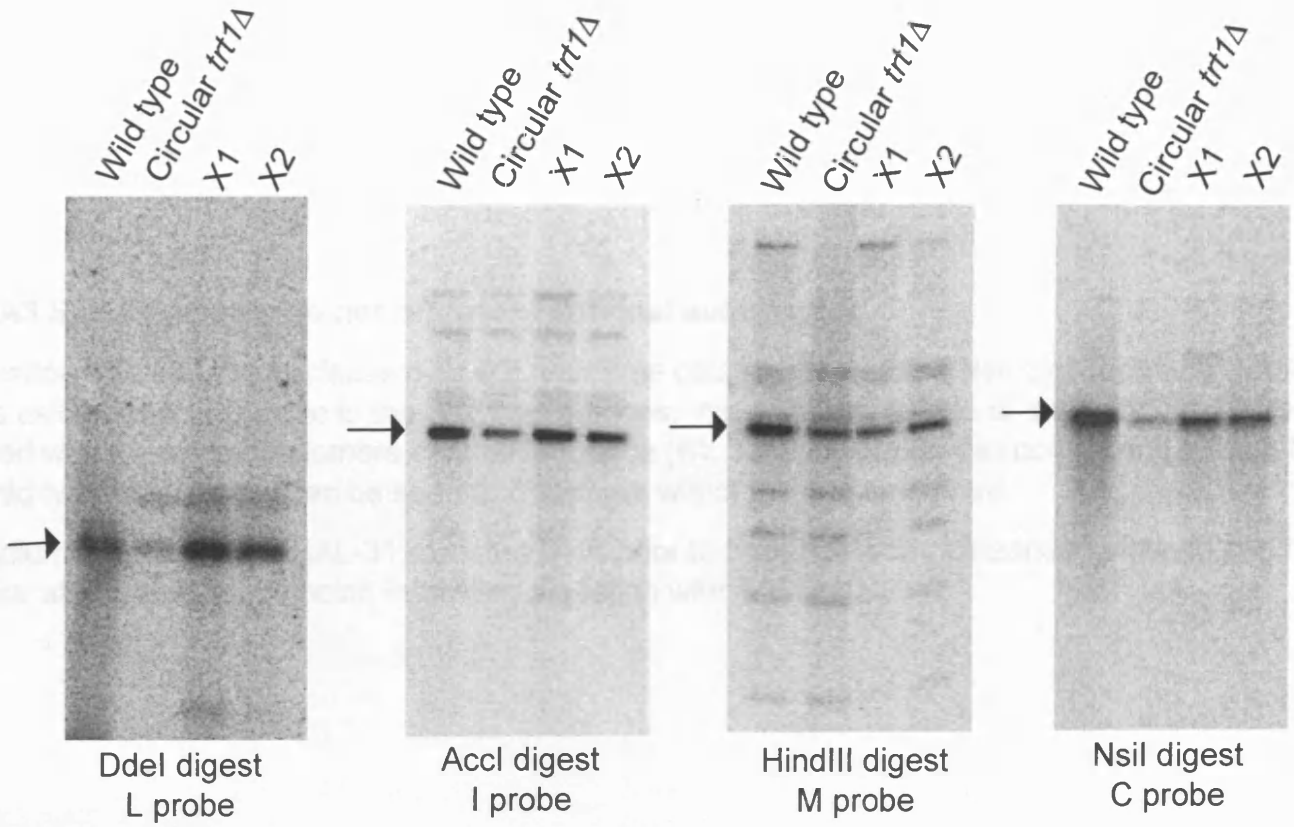


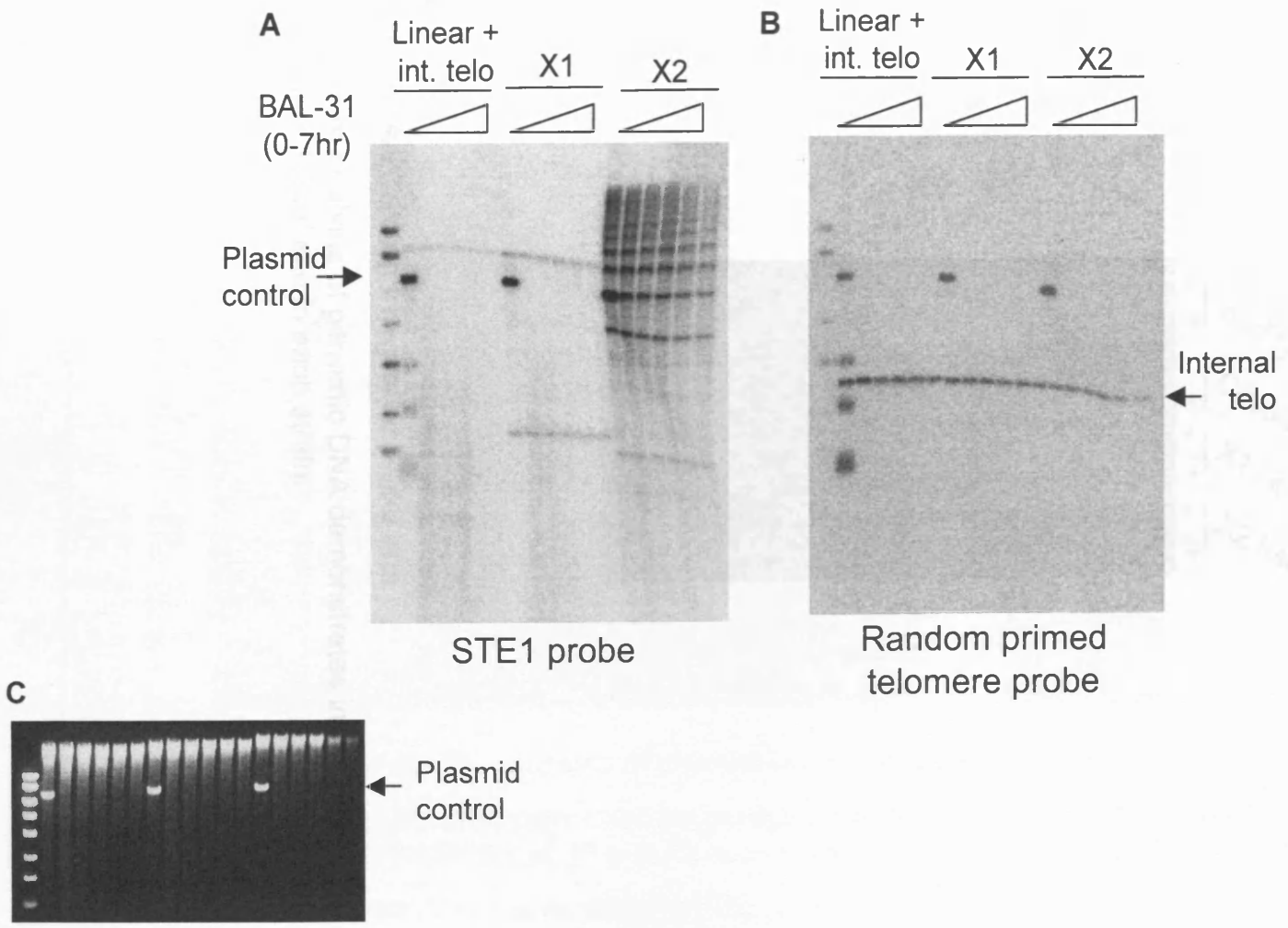
Figure A2 X1 and X2 have retained the 'LMIC' fragments

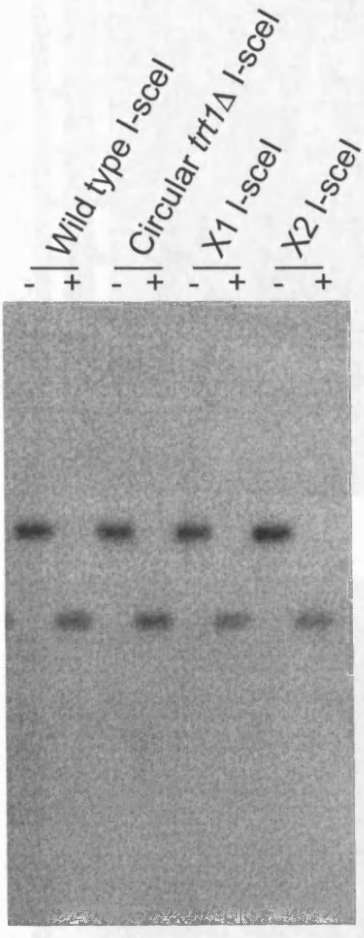
Southern anaylsis demonstrates the terminal fragments, 'LMIC' are present within the genome and have not undergone rearrangements. Arrows indicate bands of appropriate size.

Figure A3 STE fragments do not represent terminal sequences

(A) Digestion with BAL-31 nuclease over a 7 hour time course, followed by NsiI digestion and Southern analysis using a probe specific to the STE1 sequences. No decrease in size or intensity can be seen compared with the internal telomere control sequence (B). Linearised plasmid control and terminal fragments in the wild type linear strain can be seen to disappear within the first time point.

(C) Ethidium bromide gel of BAL-31 digested DNA prior to NsiI digestion. Linearised plasmid can be seen to disappear after the first time point, indicating digestion with BAL-31


A
Linear +
int. telo
X1
X2
BAL-31
(0-7hr)
Plasmid
control
STE1 probe
B
Linear +
int. telo
X1
X2
Internal
telo
Random primed
telomere probe
C
Plasmid
control



his4 probed

Figure A4 Cutting integrated I-SceI site

Southern analysis of genomic DNA demonstrates integration of an active I-SceI site in each strain.

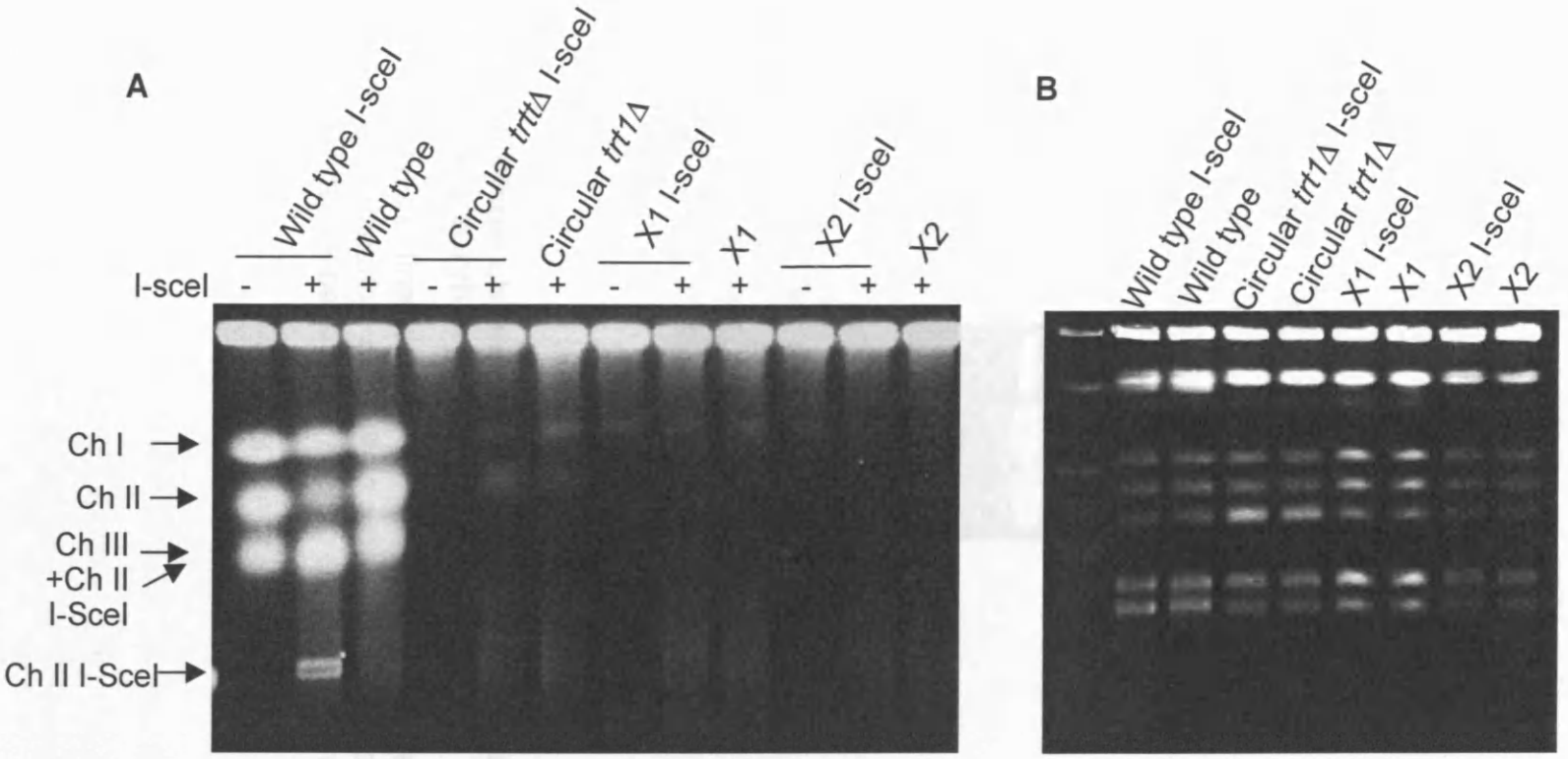


Figure A5 Pulsed field gel electrophoresis of chromosomes digested with I-SceI

(A) Chromosome II is cut. Two fragments enter the gel in a linear strain, one in a circular strain as the chromosome is linearised. Treatment of X1 and X2 does not allow entry into a pulsed field gel.

(B) NotI digestion of chromosomes shows equal loading in (A).

Figure A6 Whole chromosome pulsed field gel electrophoresis of linear *trt1*Δ survivor

Chromosomes of linear *trt1*Δ survivors, maintaining telomeres through recombination, are able to enter a whole chromosome pulsed field gel. A band is not seen to enter for chromosome III.

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